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'Doctor in Medical Sciences'
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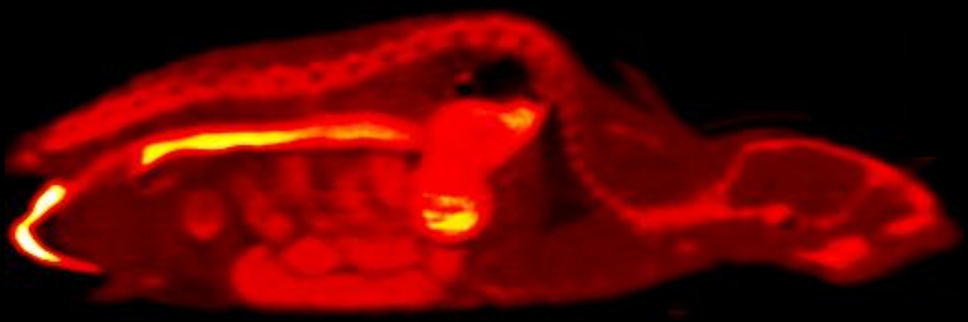
"Metallothioneins as modulators of intestinal inflammation and potential therapeutic targets for inflammatory bowel diseases"



UNIVERSITEIT GENT

Faculty of Medicine and Health Sciences
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Cover pictures:

Top left and right: metallothionein structure, copied from:

http://www.cf.ac.uk/biosi/staffinfo/kille/Lecturers/FishAdaptationslides/sld019.html

Centrefold: representative microSPECT/CT image of a mouse injected with Indium–labelled anti-MT1/2 antibody (clone UC1MT) during dextran sulphate sodium-induced colitis, yellow colour indicates radioactivity

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Abbreviations

APC	**Antigen presenting cell**
BMDM	**Bone-marrow derived macrophages**
CD	**Crohn's disease**
CT	**Computed tomography**
DAMP	**Damage-associated molecular pattern**
DC	**Dendritic cell**
DMOG	**Dimethyloxalylglycine**
DSS	**Dextran sulphate sodium**
GM-CSF	**Granulocyte-macrophage colony-stimulating factor**
H&E	**Hematoxylin and eosin**
HIF	**Hypoxia-inducible factor**
HMGB	**High mobility group box**
HSP	**Heat shock protein**
IBD	**Inflammatory bowel diseases**
IEC	**Intestinal epithelial cell**
IFN	**Interferon**
IL	**Interleukin**
IP	**Intra-peritoneal**
IR	**Intra-rectal**
IRF	**Interferon regulatory factor**
KC	**Keratinocyte chemoattractant**
KLF	**Krüppel-like factor**
LDH	**Lactate Dehydrogenase**
LPS	**Lipopolysaccharide**
MAMP	**Microbe-associated molecular pattern**
MPO	**Myeloperoxidase**
MT	**Metallothionein**
MT-KO	**Metallothionein knockout**
NF-κB	**Nuclear factor-κB**
NLR	**Nucleotide-binding oligomerization domain-like receptor**

NOD	**Nucleotide-binding oligomerization domain**
PAMP	**Pathogen-associated molecular pattern**
PHD	**Prolyl hydroxylase domain**
PRR	**Pattern recognition receptor**
RAGE	**Receptor for advanced glycation endproducts**
ROS	**Reactive oxygen species**
SPECT	**Single photon emission computed tomography**
STAT	**Signal transducers and activators of transcription**
TLR	**Toll-like receptor**
TNBS	**Trinitrobenzene sulfonic acid**
TNF	**Tumour necrosis factor**
TREM	**Triggering receptor expressed on myeloid cells**
UC	**Ulcerative colitis**
VEGF	**Vascular endothelial growth factor**

Summary

Inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are chronic inflammatory disorders of the gastro-intestinal tract. Both diseases are characterized by intestinal epithelial barrier disruption and are driven by an exaggerated immune response to luminal antigens in genetically susceptible individuals. During intestinal inflammation, oxygen supply towards the epithelium is insufficient and adaptive mechanisms are activated. This involves the induction of hypoxia-inducible factor 1 (HIF-1) which trans-activates neo-angiogenic genes such as vascular endothelial growth factor (VEGF). Parts of the epithelium get however severely compromised and these cells will release 'damage-associated molecular patterns' (DAMPs or danger signals) to attract, alarm or activate immune cells in order to inform the immune system of the damage. The attraction and activation of immune cells during intestinal inflammation includes the recruitment and polarization of macrophages towards efficient pro-inflammatory M1 cells. The M1 macrophages are essential for proper clearance of tissue debris and foreign material and further drive inflammation by the production of pro-inflammatory cytokines. However, they may cause substantial bystander damage and are therefore controlled by regulatory M2 macrophages which dampen M1 signals and initiate recovery by promoting wound healing.

Metallothioneins (MTs) are 6-7 kDa, cysteine rich proteins with cellular and immune-related functions. The MT1 and MT2 isoforms serve as zinc chaperones within the cell and through the body, protect against heavy metal toxicity and are able to capture free radicals through zinc exchange. Metallothionein 1/2 are rapidly up-regulated during stress, including inflammation, and are therefore denoted as acute stress or phase proteins. They are able to alter immune responses through the modulation of humoral and cellular immune processes and are involved in a number of inflammatory conditions. However, their role in IBD was formerly unclear. Reports in literature describing MT1/2 expression in human IBD patients are inconsistent and groups investigating the contribution of MT1/2 to intestinal inflammation did not produce conclusive results.

The general aim of this thesis was to unravel the role of MT1/2 in experimental colitis. This work consists of three chapters, each describing a specific aspect of how MT1/2 might be involved in the pathogenesis of intestinal inflammation.

In chapter 1 we describe the relation between MT1/2 and HIF-1α, the key player in the hypoxic adaptive response. HIF-1α is stabilized during hypoxia, translocated to the nucleus to form a functional HIF-1 transcription factor which induces the transcription of pro-angiogenic genes such as VEGF. HIF-1 is protective in murine IBD models and a positive correlation between HIF-1 and MT expression has been described in other inflammatory conditions. Surprisingly, we found a down-regulation of MT1/2 expression following HIF-1α stabilization *in vitro* in HT29 cells, *ex vivo* in isolated epithelial cells from human intestinal biopsies and *in vivo* in mouse colonocytes. This MT1/2 down-regulation was HIF-1 regulated, as demonstrated by siRNA targeting HIF, and was mediated through zinc. Conversely, the up-regulation of MT1/2 suppressed and MT1/2 silencing enhanced HIF-1α stabilization following prolyl hydroxylase domain inhibition. The inverse relation between MTs and HIF-1 was confirmed in dextran sulphate sodium (DSS)-induced colitis. The mRNA expression of *Mt1* was significantly induced and VEGF expression reduced during the peak of inflammation.

In chapter 2, we investigated the role of MT1/2 in experimental colitis by genetic deletion of *Mt1* and *Mt2* and by anti-MT1/2 antibodies. We showed that lacking MT1/2 significantly favours the outcome of DSS-induced colitis. Anti-MT1/2 antibody treatment partly reduced the signs of colitis and resulted in less macrophage infiltration in DSS- and trinitrobenzene sulfonic acid (TNBS)-induced colitis. We demonstrated that the applied anti-MT1/2 antibodies targeted the colon during colitis by subjecting mice, injected with radioactive antibody, to micro-single-photon emission computed tomography (μSPECT/CT). Radio-activity of the colon was significantly increased during acute inflammation and decreased during recovery. Since antibodies act outside the cell, we needed to confirm the cellular release of MT1/2. We demonstrated that MT1/2 are released upon necrotic cell death and that they are able to attract leukocytes upon release. These results indicate that MT1/2 act as danger signals, propagating intestinal inflammation.

In chapter 3, we explored the effect of MT1/2 on macrophage phenotype and polarization using bone-marrow derived macrophages from MT1/2 knockout (MT-KO) and wild type (WT) mice. The absence of MT1/2 decreased the levels of M1- and enhanced the levels of M2-associated signal transducers and transcription factors in macrophages. Subsequently, the M2 phenotype of MT-KO macrophages resulted in an enhanced response towards M2 stimulation and a lower induction of pro-inflammatory cytokines following M1 stimulation compared to WT macrophages. These results show that MT1/2 deletion shifts macrophages towards the regulatory M2 phenotype which may additionally have contributed to the observed reduced susceptibility to colitis of MT-KO mice.

Samenvatting

Inflammatoire darmziekten (IBD), de ziekte van Crohn en ulceratieve colitis, zijn chronisch aandoeningen van het gastro-intestinaal kanaal. Beide ziekten worden gekenmerkt door een beschadigde darmbarrière en een overdreven reactie van het immuun systeem tegen luminale antigenen in genetisch belaste personen. Tijdens inflammatie is de mucosale zuurstofvoorziening onvoldoende en adaptieve mechanismen worden aangesproken. Deze omvatten de inductie van hypoxie-induceerbare factor 1 (HIF-1), wat resulteert in de transactivatie van pro-angiogene genen zoals vascular endothelial growth factor (VEGF). Echter, een deel van het epitheel is zodanig beschadigd en zal door de vrijstelling van 'damage-associated molecular patterns' (DAMPs of 'danger signalen'), welke immuuncellen alarmeren, aantrekken en/of activeren, zelf de immuunrespons onderhouden. De aantrekking en activatie van inflammatoire cellen omvat de infiltratie van macrofagen en hun polarisatie naar inflammatoire M1 cellen. Deze M1 macrofagen zijn essentieel voor het fagocyteren van weefselresten en vreemd materiaal. Echter, door de productie van pro-inflammatoire cytokines onderhouden zij verder de ontstekingsreactie en kunnen zij zelf verantwoordelijk zijn voor verdere schade aan het darmweefsel. Daarom worden ze onder controle gehouden door regulatoire M2 macrofagen, welke herstel bevorderen.
Metallothioneines (MTs) zijn 6-7 kDa, cysteine-rijke eiwitten. De isovormen MT1 en MT2 fungeren als zink chaperones, beschermen tegen zware metalen en kunnen zuurstof radicalen capteren. Doordat MT1/2 snel worden op-gereguleerd bij stress en inflammatie, worden zij beschouwd als acute stress of fase eiwitten. Deze eiwitten zijn verder in staat de humorale en cellulaire immuunrespons te beïnvloeden en hun functies worden in verband gebracht met verschillende inflammatoire aandoeningen. Echter, de rol van MT1/2 in IBD was tot voordien onduidelijk. Er werden tegenstrijdige gegevens gepubliceerd omtrent de expressie van MT1/2 in IBD patiënten en hun effect op intestinale inflammatie.

Het hoofddoel van dit werk was het bestuderen van de rol van MT1/2 in experimentele darminflammatie. Deze thesis bestaat uit drie delen. Elk deel behandelt specifieke mechanismen betrokken in de pathogenese van darminflammatie en hoe MT1/2 hierin betrokken zijn.

In hoofdstuk 1 van de thesis werd de relatie tussen MT1/2 en HIF-1α onderzocht. HIF-1α wordt gestabiliseerd tijdens hypoxie wat resulteert in de inductie van HIF-1. HIF-1 is protectief in muismodellen voor IBD en in andere inflammatoire aandoeningen werd een positief verband aangetoond tussen HIF-1 en MTs. Wij vonden echter dat MTs worden neer-gereguleerd bij HIF-1α stabilisatie *in vitro* in HT29 cellen, *ex vivo in* geïsoleerde epitheelcellen van colon biopten en *in vivo* in primaire muis colonocyten. Deze MT1/2 neer-regulatie was HIF-1α gemedieerd en afhankelijk van zink. Omgekeerd, de inductie van MT1/2 resulteerde in een verminderde stabilisatie van HIF-1α. De omgekeerde verhouding tussen HIF-1α en MTs werd bevestigd in dextraan sulfaat sodium (DSS)-geïnduceerde colitis waarbij Mt1 expressie werd geïnduceerd en VEGF expressie onderdrukt tijdens actieve inflammatie.

In hoofdstuk 2 onderzochten we de rol van *MT1/2* in darminflammatie door gebruik te maken van muizen met een genetische *Mt1* en *Mt2* deletie (MT-KO) en wild type (WT) muizen. We toonden aan dat MT1/2 deletie de symptomen van DSS-geïnduceerde colitis vermindert. De behandeling van anti-MT1/2 antilichamen reduceerde deels de ziekteactiviteit in acute DSS- en trinitrobenzene sulfonic acid (TNBS)-geïnduceerde colitis met een duidelijk verminderde macrofaaginfiltratie. We toonden aan dat anti-MT1/2 antilichamen effectief ter hoogte van de darm terechtkomen tijdens colitis door muizen te injecteren met radioactieve anti-MT1/2 antilichamen en deze te onderwerpen aan micro-single-photon emission computed tomography (μSPECT/CT). Er werd een duidelijk verhoogde radioactiviteit waargenomen tijdens actieve colitis welke terug daalde tijdens herstel. Aangezien antilichamen extracellulair werken, onderzochten we de vrijstelling van MT1/2 uit HT29 cellen. Extracellulaire MT1/2 konden enkel aangetoond worden na necrose en waren bovendien in staat leukocyten aan te trekken. Deze resultaten wijzen erop dat MT1/2 fungeren als DAMPs in de pathogenese van colitis.

In het laatste hoofdstuk werd het effect van MT1/2 op macrofaag fenotype en polarisatie nagegaan adhv. beenmerg-gederiveerde macrofagen van MT-KO en WT muizen. We toonden aan dat MT-KO macrofagen verlaagde levels van M1 en verhoogde levels van M2-geassocieerde receptoren en transcriptiefactoren bevatten. Dit M2 fenotype in MT-KO

macrofagen ging gepaard met een verhoogde functionele M2 polarisatie en een verlaagde productie van pro-inflammatoire cytokines na M1 stimulatie in vergelijking met WT macrofagen. Deze resultaten wijzen erop dat MT1/2 deletie geassocieerd is met een regulatoir M2 type macrofagen, wat mogelijks kan hebben bijgedragen tot de verlaagde gevoeligheid voor DSS-colitis van MT-KO muizen.

Introduction and Aims

Introduction

I. The immunogenicity of the mucosal barrier in inflammatory bowel diseases

Inflammatory bowel diseases (IBD) comprise two chronic inflammatory diseases, Crohn's disease (CD) which can affect the whole gastrointestinal tract and ulcerative colitis (UC) which merely affects the colon and rectum [1]. Patients with IBD often present with abdominal pain, bloody diarrhoea, weight loss and fever. Diagnosis is based on endoscopic evaluation: patchy inflamed regions interspersed with normal mucosa, often with fistulas or stenosis, are frequently observed in case of CD whereas a continuous confluent colorectal inflammation with erosions and ulcerations is characteristic for UC. Histopathology is needed to confirm the diagnosis: inflammatory cell infiltration, loss of goblet cells, erosions and ulcerations are common for both diseases while a segmental or patchy transmural inflammation with granulomas is specific for CD and a superficial infiltrate with ulcerations, crypt distortion and abscesses are characteristic for UC [2–6]. Both diseases, CD and UC, show distinct differences in aetiology and pathogenesis but share a dysregulated immune response to the intestinal microflora in genetically susceptible individuals. Over the past years, studies have tried to elucidate the underlying immunopathological mechanisms of the impaired intestinal epithelial barrier and aberrant immune activation [7–9].

Intestinal homeostasis is basically depending on a proper intestinal barrier function and an adequate cross-talk between intestinal epithelial cells (IECs), the luminal microflora and lamina propria immune cells [8]. Intestinal barrier function is achieved by a continuous monolayer of IECs and is maintained by controlled IEC turn-over, by the formation of epithelial junctions and by the production of antimicrobial peptides and mucus by IECs, protecting the gut from luminal stressors. In addition, IECs are responsible for proper communication between the luminal microflora and lamina propria immune cells and thereby preserve, together with resident immune cells, a controlled immune status of commensal ignorance and foreign recognition [10]. Thus, IECs are topographically and functionally centralized within gut mucosal immunity. A disturbance in one of the tightly

regulated signalling pathways protecting this homeostatic equilibrium can initiate a cascade of immune responses, and thereby drive chronic inflammatory diseases such as IBD. Hence, the intestinal epithelium has evolved adaptive mechanisms which enable IECs to respond to (environmental) stressors and yet maintain the controlled communication with microflora and immune cells. However, these mechanisms may aid in perpetuating the inflammatory response if not efficiently turned off. Consequently, these mechanisms have been linked as contributors to the defective barrier function and/or dysregulated immune status in IBD. In this thesis, we discuss some of these aspects and investigated how metallothioneins (MTs), acute stress proteins, are integrated in these mechanisms.

1. The IEC as innate immune cell

1.1. Intestinal epithelial cells are homeostatic guardians

The intestinal epithelium is composed of specialized IECs, each conferring a different aspect of barrier protection and gut homeostasis. Firstly, enterocytes maintain the absorptive function by selective uptake of nutrients and fluids and exclude entrance of undesired compounds. Secondly, neuroendocrine cells are scattered as single cells deep in the crypts of the entire intestine and produce hormones and peptides. Thirdly, the secretory cells comprising goblet and Paneth cells are in charge of mucosal mucus secretion. Goblet cells produce the mucus, responsible for a bacterial gradient from outer to inner mucus layer, which is essential for epithelial cell sensing of commensals and protection against pathogens. Paneth cells are mainly located deep in the crypts of the ileum and produce antimicrobial peptides. Finally, specialized antigen sampling cells, M cells, are interspersed in the epithelium covering isolated lymphoid follicles and Peyer's patches and present sampled antigens to antigen presenting cells (APCs). Antigen presenting cells prime naïve T and B cells which become memory and effector cells and migrate via lymph vessels to finally home the intestinal mucosae and control oral tolerance [11].

Consecutive IECs are connected by epithelial junctions, adherens and tight junctions and desmosomes, and form a continuous monolayer which is the interface between the gut lumen containing foreign potential pathogens and the underlying mucosal layers. The intestinal epithelium is renewed every 2-3 days in the small intestine and every 6-7 days in

the colon by controlled IEC proliferation and cell shedding at the top of the villi and surface epithelium respectively. Any disturbance in epithelial turn-over or enterocyte function can cause a disruption of the epithelium and an uncontrolled influx of antigens into the lamina propria, resulting in immune cell activation and inflammation, a situation contributing to IBD pathogenesis [9,12].

1.2. Intestinal epithelial cell-interaction with microflora

Immune tolerance is accomplished by the adequate recognition of foreign pathogens and the ignorance of non-pathogenic compounds, among them the commensal gut-specific microflora. The IECs vouch, together with resident phagocytes (dendritic cells (DCs) and macrophages), for this proper recognition and are therefore provided by a set of pattern recognition receptors (PRRs) [10,13]. The PRRs recognize microbial components, known as pathogen-associated molecular patterns (PAMPs) or better microbe-associated molecular patterns (MAMPs), and control the basal immune status of gut's mucosa [13,14]. Microbe–sensing PRRs include Toll-like receptors (TLRs), intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), leucine-rich repeat receptors (C-type leptin receptors) and retinoid acid (RA)-inducible gene 1-like receptors and have genetically been linked to IBD pathogenesis [15]. Genetic deletion of different TLRs and NLRs increases susceptibility to experimental colitis whereas sustained PRR signalling maintains chronic colitis in mice. A basal PRR activity on IECs is essential for maintenance of the epithelial barrier function by induction of effector responses involved in cell survival, maintenance of IEC interconnections and production of antimicrobial peptides [10,13]. They furthermore enable a constant surveillance of the gut microbiota. However, binding of the ligand to its IEC-PRR triggers downstream signalling events resulting in the expression of chemokines and cytokines involved in innate immune activation. This activation is believed to be controlled by the polarized character of IECs conferring a homeostatic or activation response accomplished by as yet undefined mechanisms. Given their 'homeostatic' barrier function and their role as innate immune activators, it is presumable that a disturbance in PRR effector signalling, due to microbial misbalance, pathogen invasion or danger motifs recognition, may evoke inflammation and immune activation.

1.3. Intestinal epithelial cell-interaction with resident mucosal immune cells

Resident immune cells comprise specialized APCs, including DCs and macrophages, innate lymphoid cells and natural killer cells. Innate lymphoid cells are positioned within the epithelial layer and in the lamina propria. Intra-epithelial lymphocytes specifically respond to ligands expressed on IECs and although their precise role is not fully understood, these cells promote important barrier functions [16]. The APCs are, together with IECs, responsible for the oral tolerance which is achieved by luminal sampling in case of DCs or by phagocytosis in case of macrophages. Recent advantage using in vivo imaging revealed that, besides M cells, other specialized IECs, small intestinal goblet cells, function as transporters of luminal antigens to DCs [17]. These antigens are subsequently presented to prime T-cells. The APCs possess, as IECs, PRRs controlling the microbial-derived response. In addition to PAMP-mediated PRR signalling, PRRs are also triggered by signals released from stressed or damaged (intestinal epithelial) cells and are as such part of the IEC-innate immune cell intercommunication [18].

Conventional inflammatory signalling runs through cytokines, up-regulated and produced by inflammatory cells and IECs in response to a foreign pathogen. However in 1994, Matzinger proposed the 'danger hypothesis' implementing an innate immune response against 'danger' and not merely towards 'foreign'. Damaged cells express or release molecules that are normally hidden from the immune system, which alarm and/or activate surrounding cells. These signals are therefore called 'alarmins', 'danger signals' or damage-associated molecular patterns (DAMPs) [19]. These DAMPs activate immune cells by triggering cytokine release after receptor binding or they are involved in immune cell attraction. Inflammatory bowel diseases are characterized by a compromised intestinal epithelium and DAMPs which are released from IECs may alert/activate resident immune cells of the lamina propria. In addition, DAMPs are also released or secreted from activated immune cells and in turn activate IECs and other immune cells to cytokine release and further attraction of immune cells. The different PAMPs, DAMPs and their receptors that have been linked to IBD pathogenesis are further discussed below.

2. The cellular response of compromised IECs

2.1. The hypoxic adaptive response

The intestinal epithelium forms the interface between the richly perfused sub-epithelial mucosae and the anoxic gut lumen [20]. As such, IECs are subjected to a unique oxygen gradient and this 'physiologic hypoxia' renders them highly susceptible to any further decrease in oxygen supply, which occurs during inflammation. The mechanism anticipating to this internal hypoxia is called the 'hypoxic adaptive response' [20,21]. Prolyl hydroxylases (PHDs, PHD1, 2 and 3) degrade hypoxia inducible factor 1 alpha (HIF-1α), however, PHDs are inhibited during low oxygen tension leading to an increased stabilization of HIF-1α. After HIF-1α translocation to the nucleus and heterodimer formation with HIF-1β, HIF-1 transcriptionally induces different adaptive target genes, such as vascular endothelial growth factor (VEGF), glucose transport protein GLUT-1 and erythropoietin, which are responsible for re-establishment of epithelial vascularization [22].

In intestinal inflammation, and in IBD, oxygen supply towards the epithelium is insufficient partly due to vasculitis and increased oxygen consumption by the inflammatory infiltrate [20,22]. This results in a further increase of mucosal hypoxia with the net result of epithelial dysfunction, compromised barrier integrity and inflammation. The intestinal epithelium of IBD patients displays increased levels of HIF-1α and data from mouse models associate an increased HIF-1α stabilization with an enhanced outcome [23–27]. The latter warrants for future therapeutics able to activate this transcriptional machinery. Dimethyloxalylglycine (DMOG), a pan-PHD inhibitor designed for experimental approach, induces hydroxylase inhibition, HIF-1α stabilization and disease amelioration in murine IBD models [25,26]. The potential of more specific PHD inhibitors as treatment strategy for IBD is currently being explored and points in the direction of selective PHD1 inhibition since PHD1 is significantly over-expressed in IBD patients and PHD1 knockout mice are protected against experimental colitis [27,28].

2.2. Endoplasmatic reticulum stress

Protein synthesis is accomplished by chaperones of the endoplasmatic reticulum (ER) which stimulate protein folding, prevent protein aggregation and ensure correct transfer of folded proteins to cellular and extracellular compartments. A range of environmental and host-

related factors can activate the 'unfolded protein response' (UPR) and trigger proteostasis. The chaperone glucose-related protein (GRP) 78, known as binding immunoglobulin protein (BIP), is associated with the transmembrane sensor proteins inositol requiring enzyme 1 (IRE1), activation transcription factor 6 (ATF6) and PKR-like ER kinase (PERK) which are in quiescent state of activation. Upon mis- or unfolded protein sensing, BIP is dissociated from its sensors and liberates IRE1, ATF6 and PERK which initiates three branch-signalling and aims to resolve the ER stress [29]. The IECs have a high turn-over, specific roles in preserving immune homeostasis and are constantly exposed to microbes and toxins. It is thus not surprising that specialized IECs, Paneth cells and goblet cells, are most sensitive to ER stress and highly challenged in resolving ER stress [30,31]. Indeed, mice deficient for IRE1β, which is a unique IRE1 isoform of the digestive and respiratory tract, showed an increased susceptibility towards dextran sulphate sodium (DSS)-induced colitis [32]. Intestinal epithelial deletion of a key transcription factor in ER stress, X-box-binding protein 1 (XBP1), resulted in spontaneous ileitis with absence of Paneth cells, reduced goblet cells and marked ER stress in mice [33]. In accordance, deletion of other ER stress markers also resulted in spontaneous and/or increased susceptibility towards intestinal inflammation in mice and this has been linked to perturbations in the mucus production system. Anterior gradient homolog 2 (AGR2) protein is present in the ER of secretory IECs and is essential for intestinal mucus production by its association with MUC2, the main component of the mucus produced by goblet cells. Park et al. described the absence of MUC2 protein and intestinal mucus in AGR2-/- mice which resulted in increased susceptibility to DSS-colitis [34]. The AGR2-/- mice produced by Zhao showed abnormal goblet and Paneth cells, decreased MUC2 levels and developed spontaneous ileitis and colitis [35]. In accordance, MUC2-/- mice were also predisposed to DSS-induced colitis [36]. As for the ATF6 and PERK branch, ATF6α-/- mice reconstituted with wild type bone marrow show an impaired ER stress response and are highly sensitive to DSS colitis whereas the deletion of CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) protects mice from colitis [37,38]. The up-regulation of CHOP indicates PERK activation and induces apoptosis to eliminate cells that are unable to resolve ER stress.

Besides the number of mouse models linking ER stress to intestinal inflammation, activation of the UPR and impairment to cope with ER stress have been reported in IBD patients [31,33,39–41]. Furthermore, the UPR markers XBP1, AGR2 and Orosomucoid-like 3 (ORMDL3, which is

an ER transmembrane molecule) are genetically associated with human IBD underlining their role in human intestinal inflammation [33,42–44].

2.3. Mitochondrial stress

Mitochondria are physically and functionally in close relationship with the ER. While ER stress results in activation of the UPR, mitochondrial stress activates the mitochondrial UPR (mtUPR). The mtUPR also senses disturbance in protein homeostasis and aims at restoring this by inducing protein folding and degradation. mtUPR signalling involves the activation of CHOP and activator protein-1 (AP-1); however CHOP activation by ER- and mtUPR runs through different elements [45]. Recently, mitochondria have been implemented in the cellular danger response and associated immune activation in IBD [46,47]. Dysfunctional mitochondria induce a TLR4-mediated inflammatory response and mitochondrial reactive oxygen species (ROS) are able to activate NLRP3 [48,49]; two signalling cascades which have been shown to contribute to IBD pathogenesis. Furthermore, IECs of IBD patients show abnormal mitochondria and mtUPR is activated in mouse models for IBD [46,50,51]. Thus, future studies using mice deficient in mtUPR players will highlight the specific mechanisms underlying the role of mitochondrial stress in IBD.

2.4. The release of DAMPs

The intestinal epithelial monolayer is physically maintained by the adequate renewal of the IECs. This process is controlled by proliferation, differentiation and migration of IECs along the crypt and villus axis. Any process interfering with this fine-tuned machinery may evoke stress to the epithelium, forcing IECs to respond in a self-defensive or –destructive manner. In patients with IBD, epithelial barrier continuity is affected and different forms of (excessive) cell death have been implemented in this process. Apoptosis, or controlled cell death, is physiologically responsible for IEC shedding (anoikis) at the top of the villus and surface epithelium, which is initiated by the loss of cell-cell contact, and excessive rates of apoptosis have been observed in patients with IBD and IBD mouse models [52–57]. Apoptotic cells normally transmit ‘find-me’ signals towards phagocytes, resulting in the silent removal of apoptotic bodies without evoking an inflammatory response. However, one can assume that when the apoptotic rate exceeds phagocyte capacity, these cells end up as secondary

necrotic cells eventually bursting and spilling their intracellular content just like primary necrotic cells [58]. Necrosis refers to uncontrolled cell death characterized by plasma membrane damage. However recently, a specific form of controlled necrotic cell death 'necroptosis' has been introduced. Necrosis is inherent to any inflammatory process and necroptosis has been implemented in the pathogenesis of IBD [9,59–62]. While different regulatory pathways controlling all forms of cell death are being unravelled, its decisive result eventually defines the outcome, namely, if the cell leaks its intracellular content or not (*Figure 1*). Necrotic as well as necroptotic cells herein hold the same fate, the intracellular content will be spilled out and this contains danger signals. The release of DAMPs is most likely a natural occurring event to activate local immune cells and initiate an adequate immune response. However, during excessive cell death and signal release, DAMPs may prolong the initial inflammatory response resulting in chronic immune activation and inflammation, a situation that is actively present in IBD patients (*Figure 2*: pp. 28) [19,58,63].

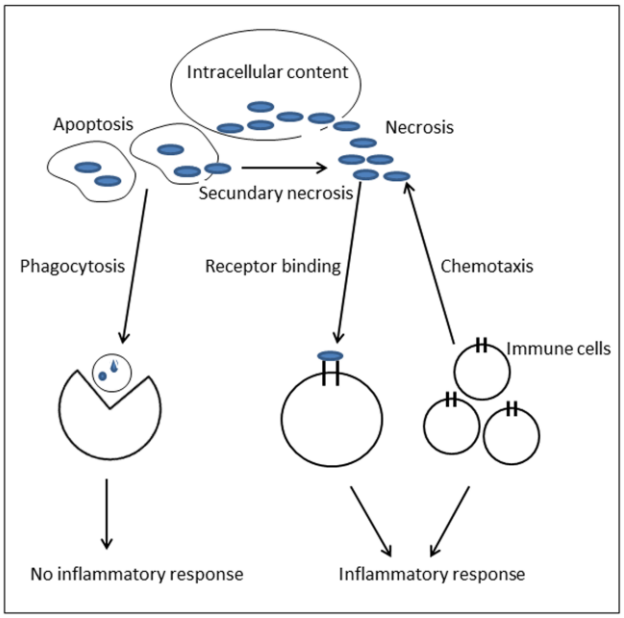

Figure 1: Effect of apoptotic and necrotic cell death on immune activation. During apoptosis, the intracellular content remains hidden from the immune system and cells are removed by phagocytosis. During necrotic cell death the integrity of the plasma membrane is compromised and intracellular DAMPs now leak outside the cell. These extracellular signals communicate with local immune cells and activate the immune system. The same occurs when apoptotic bodies are inefficiently removed, adapted from Kono and Rock, 2008 [58].

3. Pathogen- and damage-associated molecular patterns in IBD

Gut homeostasis covers a situation of 'controlled inflammation' where the host can co-exist with the multitude of microbes in the luminal gut. Current hypotheses in IBD pathogenesis are (1) a loss of tolerance against the luminal microflora, sensed as 'foreign MAMPs' and/or (2) a massive infiltration of exogenous environmental antigens due to barrier disruption which are sensed as PAMPs. Normally, the immune response following MAMP or PAMP recognition is quickly and efficiently turned off once the stimulus is removed. Patients with IBD lack the potential to switch off the initial response, resulting in chronic immune activation and inflammation. In addition, Matzinger postulated that the immune system also responds in absence of an exogenous signal but is simply turned on in case of stress and tissue damage which is mediated by endogenous activators, DAMPs [19]. Typically, DAMPs are passively released following plasma membrane leakage or are actively secreted from activated immune cells. Conventionally, PAMPs and DAMPs are sensed by PRRs. The PRRs recognizing microbial parts are well described and some of them also sense danger signals. Two specific DAMP-sensing PRRs, receptor for advanced glycation end-products (RAGE) and triggering receptor expressed on myeloid cells (TREM-1), have been identified and linked to IBD. However, for different signals functioning as alarmins, the concomitant PRR has not been identified. We discuss two families of well described PAMP- and DAMP-sensing PRRs and the best described alarmins recently discovered to function as danger signals in IBD.

3.1. Pattern recognition receptors involved in intestinal inflammation

Of the 4 PRR families, TLRs and NLRs are the best characterized and have extensively been investigated in the IBD research field. Toll-like receptors are located at the cellular and/or endosomal surface and conventionally recognize extracellular and/or engulfed MAMPs and PAMPs [14]. All TLRs signal through the adaptor molecule Myeloid Differentiation Primary Response gene (MyD88), with exception of TLR3 which signals through Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF). Toll-like receptor signalling results in the activation of the nuclear factor-κB (NF-κB) and the mitogen-activated protein kinase (MAPK) pathway which induce the expression and release of pro-inflammatory cytokines and chemokines [64,65]. The group NOD-like receptors encompasses 4 cytoplasmatic

receptors of which NOD1 and NOD2 are best characterized. Upon ligand recognition, the NF-κB and MAPK pathway are activated leading to the induction of cytokines and chemokines. Typically, NLR activation results in the formation of inflammasomes, large multiprotein complexes, which induce the active release of interleukin (IL)-1β and IL-18. Interestingly, inflammasome cytokine release depends on TLR signalling whereas NLRs in turn dampen activated TLR cascades [66–68]. Both TLR4 and NOD2 polymorphisms have been linked to IBD, bridging the results from mouse models to human disease [69–71] (see *Table 1*).

Table 1. Pattern recognition receptors involved in intestinal inflammation [14,72,73]

Receptor	Epithelial localization	Typical ligand	Role in the gut	Effect of receptor modulation in mice
TLR2 [53,74,75]	Cell surface (mainly crypt IECs)	Gram+ and Gram- compounds	Epithelial integrity (tight junctions)	Deletion: ↑ susceptibility to colitis
TLR4 [52,76–78]	Cell surface (mainly crypt IECs)	Lipopoly-saccharide	Essential for oral tolerance, colonocyte proliferation and protects against apoptosis	Deletion: ↑ susceptibility to colitis
TLR5 [79,80]	Basolateral celmembrane (mainly colonocytes)	Flagellin	Microbial recognition	Deletion: spontaneous colitis, ↑susceptibility
TLR9 [81–84]	Apical+basolateral celmembrane and endosomal	Unmethylated CpG	Apical: tolerance to TLR stimulation; Basolateral: NF-ɣB activation	Preventive activation: ↓ susceptibility to colitis; Therapeutic activation: ↑ susceptibility to colitis; Deletion: ↑ susceptibility to acute colitis and ↓ disease activity during chronic colitis
NOD1 [85,86]	Cytoplasm	Medo-diamino-pimetic acid	Normal response to gut microbiota	Deletion: ↑ susceptibility to colitis
NOD2 [66,87–90]	Cytoplasm (mainly Paneth cells)	Muramyl dipeptide	Secretion of antimicrobial peptides	Deletion: ↑ susceptibility to colitis; Overexpression: ↓ susceptibility to colitis
NLRP3 [91–93]	Cytoplasm	Muramyl dipeptide, bacterial RNA, mitochondrial DNA, crystals	Gut microbioom; Epithelial proliferation following injury	Deletion: ↑ or ↓ susceptibility to colitis
NLRP6 [94,95]	Cytoplasm		Gut microbioom	Deletion: spontaneous colitis and ↑ susceptibility to colitis

3.2. Danger signals as modulators of intestinal inflammation

Any intracellular compound can be released upon plasma membrane damage and can serve as potential endogenous danger signal. However, only for a few molecules that are released after cell death, immune modulating properties have been described. They are subdivided into constitutive and inducible signals. The constitutive ones are ever present in the cell and released upon cellular damage, the inducible signals are newly created, up-regulated or modified during cellular stress [63].

3.2.1. High-mobility group box 1

High-mobility group box 1 (HMGB1) is a nuclear protein that binds and distorts DNA to allow transcription factors to bind to chromatin. The protein moves from the nucleus to the cytosol through nuclear pores which is essential for its cellular release [96]. As prototypical DAMP, HMGB1 is passively released from necrotic cells or actively secreted from activated immune cells in response to different inflammatory triggers, probably via a non-classical pathway [18,97–100]. The protein functions as danger signal by binding to TLR2 and TLR4 and to RAGE and TREM which all signal through the adaptor MyD88 and activate NF-κB [101–104]. High-mobility group box 1 modulates the production of pro-inflammatory cytokines and DC maturation and exerts a direct chemotactic effect on neutrophils [68,101–103,105,106]. Protein levels are correlated with disease activity in murine colitis and this has recently been confirmed in human IBD patients [100,107,108]. Increased levels of HMGB1 are found in the stool of IBD patients and faecal HMGB1 levels correlated with faecal calprotectin levels, a commonly used biomarker of IBD activity [100]. In vitro assays revealed that HMGB1 is released from human Caco-cells and mucosal biopsies treated with tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Further human trails will have to reveal if blocking HMGB1's function as danger signal is able to reduce disease activity in IBD patients or if HMGB1 merely could function as novel biomarker. Preventing the release of HMBG1 was at least able to reduce the signs of colitis in mouse models for IBD [108].

3.2.2. Heat shock proteins

Heat shock proteins (HSPs) are highly conserved proteins which function as intracellular chaperones in protein processing and are important cytoprotectants. They are defined

according to their molecular weight and located in different cellular compartments. Some of them are constitutively expressed, others induced upon different stimuli such as heat shock and inflammation [109–112]. Heat shock proteins are generally referred to as danger signals released from necrotic cells [63,113,114]. However, stimulation with IFN-γ, an important pro-inflammatory cytokine, was also able to release the constitutive HSC70 from cancer cells [115]. As extracellular 'chaperokines', they stimulate the production of cytokines from APCs and the subsequent activation of T-cells has found its therapeutic application in cancer management [116]. In contrast, macrophages from HSP70 over-expressing mice showed a reduced LPS-triggered activity and less pro-inflammatory cytokine production which was translated in a decreased susceptibility to colitis in mice over-expressing HSP70 [117,118]. Heat shock proteins additively exert a cytoprotective effect on IECs through the inhibition of NF-κB signalling and consecutively reduce pro-inflammatory cytokine transcription in IECs [110]. Pro-inflammatory cytokines play an important role in the activation and infiltration of leukocytes during experimental colitis and IBD. Thus, HSPs seem to exert as mitigating mediators of intestinal inflammation through a negative feedback loop on pro-inflammatory cytokine-mediated excessive immune activation. The immunogenic adjuvant activity of HSPs exploited in cancer therapy and their opposite cytokine reducing function in murine colitis might depend on the initial trigger and the context in which these proteins act. Perhaps the intracellular protective effect of HSPs on IECs overrules their extracellular immune-stimulatory role in experimental colitis.

3.2.3. S100 proteins

The calgranulin family of proteins, S100 proteins, includes small calcium binding proteins with different intracellular functions. The proteins S100A8, S100A9 and S100A12 are passively released from necrotic cells and actively secreted from activated monocytes and they have been designated as danger signals [119,120]. They bind to TLR4 and RAGE and are involved in chemotaxis of leukocytes and S100A12 also induces pro-inflammatory cytokine secretion from macrophages [121–125]. The S100A8 and S100A9 proteins primarily form the heterodimeer S100A8-A9, which is known as calprotectin and used as biomarker in inflammatory diseases. Neutrophils are the main source of S100A8-A9 and plasma S100A8-A9 correlates well with the number of circulated neutrophils [126]. Faecal S100A8-A9 is a

valuable non-invasive diagnostic tool to evaluate disease activity and to predict flares in IBD patients [127,128].

The list of DAMPs involved in intestinal inflammation is certainly indefinite. Inflammatory bowel diseases are characterized by a breakdown of the intestinal epithelium and an excessive activation of the immune system. Thus, these 'innate' signals could represent therapeutic targets under pathologic condition where they are part of modulating the cascade of inflammation.

4. The duality of macrophages in intestinal inflammation

Macrophages originate from a hematopoietic stem cell in the bone marrow. They are released in the blood stream as monocytes and extravasate and differentiate to tissue-specific macrophages. These resident macrophages function as first responders towards an initial stimulus and induce neo-synthesis of bone-marrow derived monocytes and recruitment of these inflammatory cells from the blood to the inflammatory region. Generally, macrophages are divided into different subset according to their response following cytokine stimulation. The classically activated macrophages, called M1 types, are responsible for host defence and are activated by PRR ligands such as lipopolysaccharide (LPS) and IFN-γ. Upon activation, they typically secrete pro-inflammatory cytokines, chemokines and adhesion molecules. The alternatively activated macrophages, M2 types, have anti-inflammatory functions and are regulated by IL-4 and IL-13. Inflammatory M1 macrophages are essential for the antimicrobial defence but can additionally cause bystander tissue damage and must therefore be controlled by regulatory and anti-inflammatory M2 macrophages, which drive wound healing and tissue restoration [129].

In the gut, resident mucosal macrophages play a central role in detection and digestion of pathogens, toxins and cell debris and are, on the other hand, responsible for the ignorance of self-antigens and oral tolerance. Disturbance in macrophage subsets may likely challenge the controlled status of immune activation in the gut. Colonic resident macrophages house in an anti-inflammatory state, which is regulated by IL-10 levels. Different groups have

focused on the mechanisms disturbing this balance and the contribution of different macrophage subtypes to intestinal inflammation. Kühn et al. showed that interference in IL-10 signalling results in chronic gut inflammation as demonstrated in mutant IL-10 knockout mice and Li and colleagues recently showed that this can be attributed to a macrophage-specific effect using macrophages with a selective deletion of IL-10Rα [130,131]. Platt et al. showed that TLR+ macrophages that produce TNF are recruited in a CCR2 dependent manner during colitis and that these macrophages promote inflammation whereas TLR- macrophages present a different subset of macrophages, independent of inflammation. The CCR2 knockout mice that were unable to recruit these pro-inflammatory macrophages were protected from colitis [132]. Recently, an M1/M2 imbalance has been linked to experimental colitis whereas M2 alternative macrophages conferred protection and substitution of M2 macrophages reduced colitis in mice [133,134].

The contribution of different macrophage subsets to IBD is far from being fully unravelled. Patients with CD possess a specific subset of CD14+ resident macrophages which have an increased production of pro-inflammatory cytokines. However, IBD patients seem to have an impaired primary response against locally injected pathogens due to failure of TNF secretion from pro-inflammatory macrophages [135,136]. Additionally, M2 macrophages are involved in the initiation, propagation and resolution of fibrosis. Fibrosis results from tissue repair but causes intestinal strictures in CD patients when insufficiently turned off. It is not unlikely that in multifactorial diseases such as IBD, specific macrophage subsets may have differential roles depending on genetic as well as environmental factors. Furthermore, plasticity among macrophage types has been described and a specific phenotype with an associated role at onset of inflammation may switch towards an opposite macrophage type at a later stage of disease. Therefore, mediators which are able to polarize macrophages into a specific subtype may have valuable therapeutic potential at specific time points during disease progression.

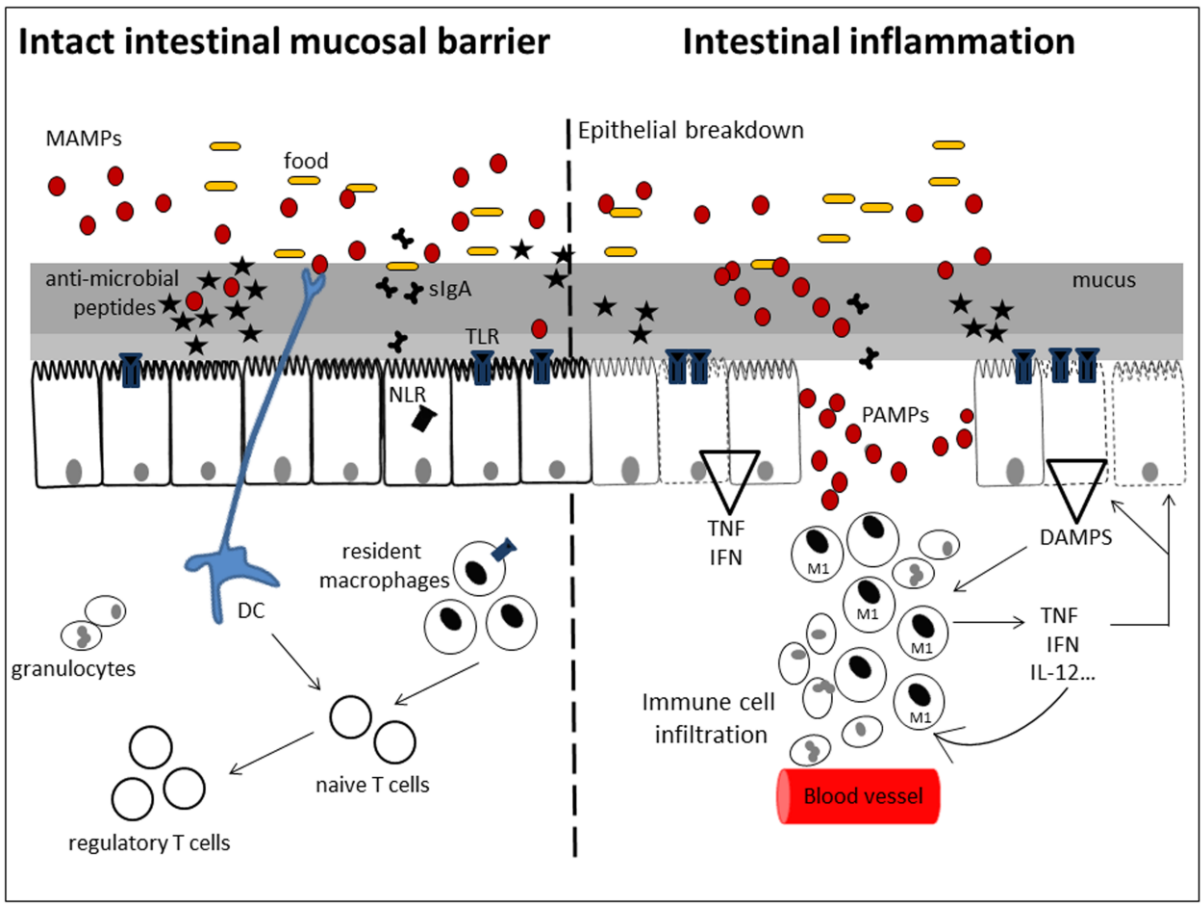

Figure 2: Intestinal mucosal homeostasis and its breakdown during inflammatory bowel disease. Intestinal homeostasis is accomplished by an intact monolayer of intestinal epithelial cells (IECs) and an adequate crosstalk between IECs, luminal microflora and resident lamina propria immune cells. During intestinal inflammation, the intestinal epithelial barrier is compromised and IEC-derived cytokines and danger signals are released and activate and attract immune cells. In addition, luminal constituents penetrate into the lamina propria and further activate immune cells to pro-inflammatory cytokine production. DAMP: damage-associated molecular pattern; DC: dendritic cell; IFN: interferon; IL: interleukin; MAMP: microbe-associated molecular pattern; NLR: NOD-like receptor; PAMP: pathogen-associated molecular pattern; TLR: Toll-like receptor; TNF: tumour necrosis factor. Adapted from Hindryckx and Laukens 2012 [137].

II. The use of chemically induced mouse models for IBD

Animal models are essential in the progression towards the understanding of disease pathogenesis. While the mechanisms underlying IBD onset and progression are complex, animal models are designed to mimic immune-pathological characteristics of these diseases. Employed models include spontaneous, genetically engineered, immune modulating (adoptive transfer) and chemically induced colitis models. The latter encompasses dextran sulphate sodium (DSS)- and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis; due to their simplicity, low cost and reproducibility they are the two most widely used models for intestinal injury. If the drawbacks inherent to the models are well considered, these models can provide valuable information about the pathogenesis and therapeutic options of IBD patients.

1. Dextran sulphate sodium-induced colitis

Dextran sulphate sodium is a sulphated polysaccharide that induces colonic inflammation when administered through drinking water in rodents [138–141]. The extend, severity and duration of inflammation depend on the dose and molecular weight of DSS, the duration and frequency of administration, the mouse strain and the experimental (microbial) environment [142–146]. The administration of 2-5% DSS (MW of 40 kDa), administered for a period of 5-7 days, is most commonly used and induces acute mid-to-distal colitis. Since this application results in colonic erosions and a cytokine profile associated with both a Th1 and Th2 response, it is often referred to as model for human UC. Indeed, when applied as 'mucosal wound healing' model, it resembles active flares of UC patients with diarrhoea and rectal bleeding, weight loss and severe illness. Histology shows a superficial inflammation with epithelial erosions, goblet cell depletion and (sub)mucosal polymorphonuclear and mononuclear leukocyte infiltration. This model is thus suitable for studying epithelial renewal and innate immunity involved in the onset and progression of acute colitis. The cyclic administration of DSS and water is conveniently applied to mimic the relapsing character of chronic UC and to assess the involvement of the adaptive immune system. Repeated cycles of DSS/water mimic chronic injury-induced inflammation with a predominant Th2 cytokine profile. Histology reveals alterations of inflammatory regions and

epithelial repair with mononuclear cell infiltration and lymphoid follicles in some cases; which is however more characteristic for colonic CD than UC [138,144,147,148]. Chronic DSS administration can additionally be used to induce dysplasia, a common feature in the course of chronic human UC [149].

A huge gap in the use of DSS is the nescience of the trigger initiating exclusively colonic inflammation. The mucus composition substantially differs between the colon and the ileum and this may define DSS preference to colonic injury [150]. Indeed, during DSS-induced colitis bacteria are in close contact with the epithelium which is normally protected by the impermeable colonic mucus layer. The same alterations in mucus composition and bacterial exposure are present in UC patients compared to controls and patients in remission [151,152]. Increased mucosal permeability and loss of crypts, followed by epithelial cell death and erosions, appear to be the earliest signs post DSS administration. Inflammation is believed to be secondary to the epithelial damage [153]. Direct damage of DSS to epithelial cells has been shown [154]. However, DSS can already be found in colonic macrophages one day after DSS administration so it is unlikely not to cause any immune cell activation following epithelial penetration and macrophage uptake [144]. An additional factor anticipating in DSS-induced colonic injury is that the bacterial load of the colon surpasses that of the small intestine with a factor 10^4 [155]. Bacteria determine DSS-induced colitis and intestinal inflammation in general and this recently resulted in human treatment in extremis: the transplantation of life companions 'healthy microflora' to patients presenting with signs of dysbiosis! A final consideration when using the acute DSS-model for IBD, a disease characterized by a dysregulated immune-response, is that adaptive immunity is not required to induce colitis. Mice lacking functional immune cells of the adaptive immune system, for example SCID and Rag mice, readily develop colonic inflammation following DSS so T and B cells are not required for colitis induction. Nonetheless, lymphocytes are essential to recover so these mice are unlikely suitable to study epithelial repair [156]. When focusing on adaptive immune processes involved in the chronicity of disease, repeated cycles of DSS can be applied; however, other immune-modulating models such as the adoptive transfer model might be more suitable.

2. Trinitrobenzene sulfonic acid-induced colitis

Trinitrobenzene sulfonic acid is a hapten that is applied intra-rectally (IR) in combination with ethanol to induce distal colitis in rats and mice. Ethanol is required to break the mucosal barrier whereas TNBS is believed to haptenize (colonic or microbial) proteins and induce a delayed-type hypersensitivity (DTH). This DTH requires TNBS pre-sensitization which is often not implemented in the protocol. Basically, the TNBS model can be applied in three different ways, depending on the objective of the study: (1) a single IR bolus of TNBS/EtOH results in an acute transmural inflammation with epithelial erosions, leukocyte infiltration and granuloma formation which histologically resembles colonic CD, (2) when the IR bolus of TNBS/EtOH is preceded by TNBS pre-sensitization (via skin or IR application), a specific DTH Th1-driven response is generated which is used to mimic the auto-immune character of CD and (3) weekly IR administrations of TNBS/EtOH result in chronic lesions associated with repeated DTH reactions and fibrosis, corresponding to the chronic aspect and complications of CD. The cytokine profile resembles a Th1 response, both in acute and chronic TNBS-induced colitis [145,147,157,158]. Remarkable, oral administration of TNBS simultaneously with IR TNBS instillation completely prevents TNBS-induced colitis [159–161]. Additionally, T cells from mice with TNBS colitis respond to own self-microflora which further strengthens the involvement of the loss of tolerance to the mucosal microflora in this model [161,162]. The administration of mucosal T cells from mice with TNBS-colitis to recipient mice induces colonic inflammation whereas simultaneous administration of anti-IL-12, a central cytokine in Th1 T cell differentiation, completely prevents colitis induction [161,163,164]. Thus, the TNBS model with pre-sensitization is highly suitable to study T cell dependent immune mechanisms involved in IBD.

The sensitivity to TNBS highly depends on the mouse strain. Literature describes the sensitive SJL/J and BALB/c mice whereas C57BL/6 and C57BL/10 mice seem to be more resistant [159,165]. However, both successful as lethal TNBS experiments have been reported in C57BL/6 mice, which is in line with our own observation [157,166,167]. Basically every individual setting requires the optimization of TNBS concentration to generate the desired disease activity in the chosen mouse strain, and this in fact also relates to DSS-induced colitis. The advantage of TNBS to DSS involves the possibility of exploring adaptive immune-mediated mechanism and the loss of oral tolerance, two important aspects in the pathogenesis of IBD.

III. Metallothioneins and intestinal inflammation

1. Metallothioneins

1.1. Structure

Metallothioneins (MTs) are 6-7 kDa proteins, initially isolated from the horse renal cortex in 1957 by Margoshes and Vallee [168]. The mammalian protein is formed of 61 to 68 amino acids with 20 of them being cysteines. The position of the cysteines is highly conserved and arranged in specific motifs: Cys-X-Cys, Cys-Cys-X-Cys-Cys, Cys-Cys-X-Cys (*Figure 3*) [169–171].

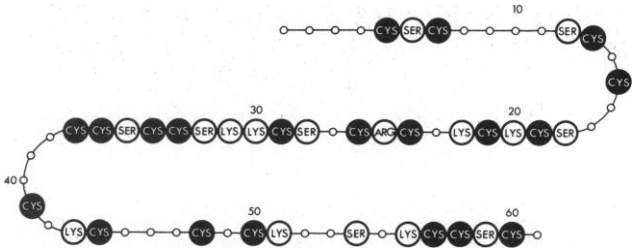

Figure 3: Amino-acid sequence of MT1B from the equine kidney, copied from Kojima et al,. 1976 [170].

All cysteine residues are involved in metal ligation through thiolate binding, bridging each metal ion to another in a two cluster formation. The cluster to the N-terminal, the β domain, consists of nine cysteines and is able to bind 3 metals; the C-terminal α domain contains 11 cysteines and binds 4 metal ions (*Figure 4*) [172–176].

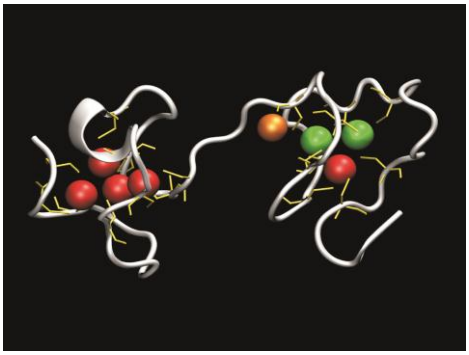

Figure 4: Spatial structure of human MT1A. Cysteine residues are shown as yellow sticks, ions are shown in red (Cd2+), green (Zn2+), and orange (Na+), copied from Laukens et al., 2009 [177].

Mammalian MTs are subdivided into 4 isoforms, MT1 to MT4. The genes are located on a single chromosomal locus on chromosome 16 for human MTs and chromosome 8 for murine Mts. The human MT1 isoform has several functional subforms (MT1A, MT1B, MT1E-H,

MT1M and MT1X) and different pseudogenes are reported as well. For human MT2 only 1 isoform, MT2A, has been identified. Murine Mts are less divergent without subdivisions for any isoform (*Mt1*, *Mt2*, *Mt3*, *Mt4*) [177–183]. Importantly, MT1 and MT2 are highly similar (*Figure 5*) whereas MT3 and MT4 are quite distinct and this is reflected in the similar tissue distribution and function of MT1/2 compared to MT3 and MT4.

Mt1: mdpncscstg gsctctssca cknckctsck ksccsccpvg cskcaqgcvc kgaadkctcc
Mt2: mdpncscasd gscscagack ckqckctsck ksccsccpvg cakcsqgcic keasdkcscc

Figure 5: protein sequence of murine metallothionein (Mt) 1 (NCBI Reference Sequence: NP_038630.1) and Mt2 (NCBI Reference Sequence: NP_032656.1). Different amino acids are indicated in red.

1.2. Expression

The MT1 and MT2 isoforms are constitutively expressed in almost every tissue. However, expression levels vary according to the tissue, cell type and the species. The highest concentrations are found in the liver, kidney, gut and pancreas. In the intestine, MT1/2 are mainly expressed in intestinal epithelial cells, with a higher expression in the ileum compared to the colon (unpublished data, *Figure 6*).

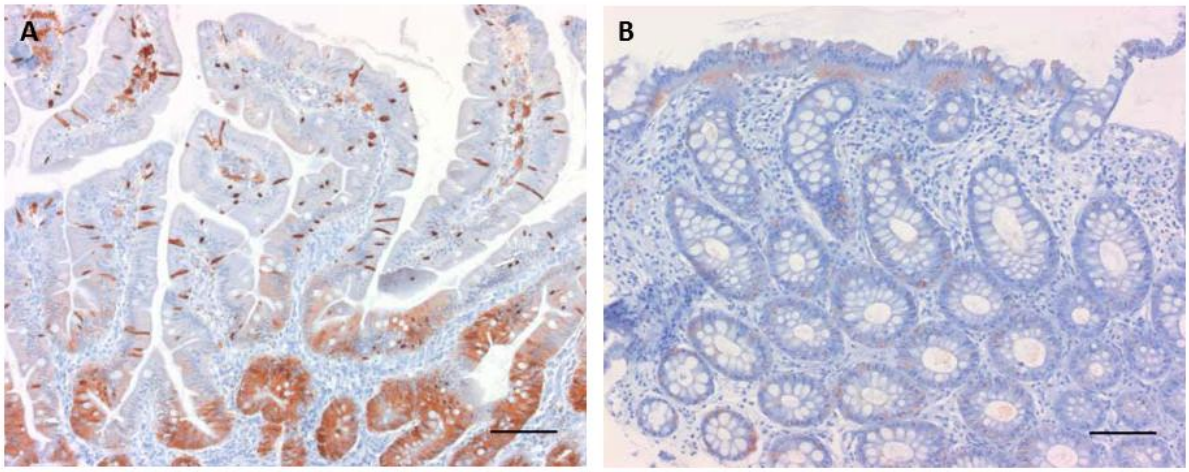

Figure 6: Immunohistochemical staining for MT1/2 on an ileum (A) and colon (B) section of a healthy subject. Scale bars: 100 µm.

Differences in MT1/2 concentrations among species have been reported for the liver. The human liver contains up to 400-700 µg/g liver whereas in rodents this is only 2-10 µg/g liver. The expression of MT1/2 is highly inducible upon different stimuli such as metals, pro-inflammatory cytokines and other chemicals which induce a stress or inflammatory reaction. As such, MT1/2 are considered 'acute stress proteins' (see further) [183].

Metallothionein 3 is considered as the brain-specific isoform and its function has mainly been investigated in neuronal disorders. However, mRNA levels of MT3 have been found in the heart, kidneys, reproductive organs and recently, we showed that MT3 is clearly expressed in mouse intestinal tissue albeit to a lesser extend than MT1 and MT2 (unpublished data, see future perspectives). In contrast to MT1/2, MT3 is not induced by metals or pro-inflammatory stimuli [184–186]. Metallothionein 4 is only expressed in stratified squamous epithelia and maternal deciduum and is, as for MT3, not induced by zinc or pro-inflammatory stimuli [182,187].

The ubiquitous nature of MT1 and MT2 and their conserved aspect suggest an important role for these proteins. Despite the fact that mice with a genetic deletion of *Mt1* and *Mt2* have a normal growth and phenotype under physiologic conditions, *in vivo* experiments using MT mutant mice indicate that MT1 and MT2 are important in a broad range of stressful conditions, including immune-driven pathologies. We focused on MT1 and MT2 and further refer to these isoforms through this dissertation.

1.3. Biological properties of MT1 and MT2

1.3.1. Metallothioneins as metal binding proteins

The best known characteristics of MT1 and MT2 came along with their identification as cadmium and zinc-containing proteins [168,169]. Since then, MT's metal binding capacity has been subject of many research. The fact that MT1/2 are able to exchange essential trace elements such as zinc and copper to other metalloproteins suggested a function as metal reservoir and preserver of metal homeostasis within the cell and through the body. The high inducibility of MT1/2 upon metal exposure *in vitro* as well as *in vivo* indicated a bi-partial metal binding function, namely also providing protection against heavy metal toxicity [188–190]. The latter has extensively been explored following the development of mice lacking functional *Mt1* and *Mt2* genes [191,192] (further referred to as MT knockout mice, MT-KO mice)

and mice overexpressing *Mt1* [193] which initially revealed an increased sensitivity of MT-KO mice upon heavy metal exposure [194,195]. Certainly, the development of these mutant mice opened the path to the exploitation of the role of MT1/2 under physio- and pathological condition in an *in vivo* biological model.

1.3.2. Metallothioneins as anti-oxidantia

Metallothioneins have a nucleophile character and can bind ROS through ion exchange [196,197]. A number of *in vitro* studies demonstrated anti-oxidant capacity of MT1/2 by oxidation of MT upon ROS or radical sequestering and Zn^{2+} release [198–202]. *In vivo*, MT1/2 protect in situations associated with ROS production whereas lacking functional *Mt1* and *Mt2* genes sensitized against oxidative stress. For example, *Mt1* overexpressing mice were protected against ischemia-reperfusion induced myocardial injury [203,204] whereas cells isolated from MT-KO mice showed an increased sensitivity to oxidative stress [205]. This property has further been explored in the light of cancer therapy where MT1/2 conferred protection against electrophilic antineoplastic agents [206].

1.3.3. Metallothioneins as acute stress proteins

The synthesis of MT1 and MT2 is rapidly induced by metals, cytokines, hormones, chemicals, stress and inflammation through metal responsive elements (MRE), anti-oxidant responsive elements (ARE) and in some MT genes, glucocorticoid responsive elements (GRE) (*Figure 7*). However, other responsive elements in the MT1 and MT2 promotor region have also been detected to respond to inflammatory mediators. Only two transcription factors have been shown to influence metal-induced MT1/2 transcription, MREBP (MRE binding protein that binds MRE of hMT2A) and MTF-1 (MRE binding transcription factor) [207–209]. The transcription factor MTF-1 is thought to be essential for MT1 and MT2 expression since MTF-1 knockout cells show no MT1 and MT2 levels, neither at basal level nor after metal treatment [210]. Interestingly, MTF-1 is also responsible for the regulation of other, vitally important, genes since MTF-1 knockout mice die in utero [211]. Notable, human MT1 and MT2 transcription is regulated independently whereas mouse *Mt1* and *Mt2* are coordinately regulated [212,213].

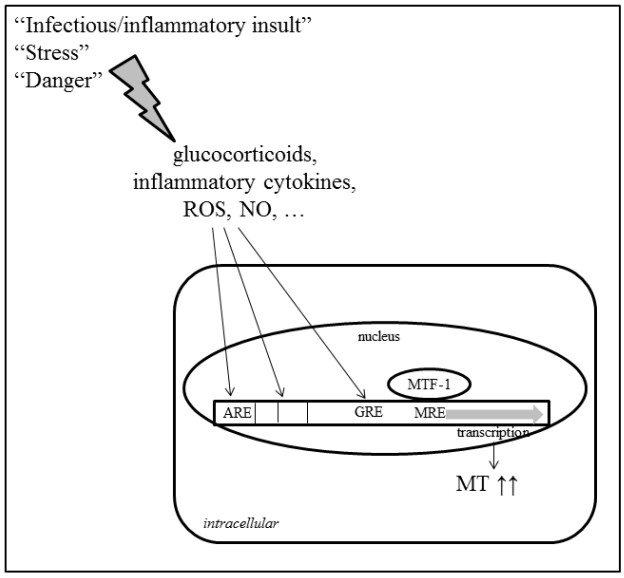

Figure 7: Simplified representation of stimuli inducing metallothionein (MT) 1 and MT2 expression. ARE: anti-oxidant responsive element; GRE: glucocorticoid responsive elements; MRE: metal responsive element, MTF-1: metal transcription factor-1; NO: nitric oxide; ROS: reactive oxygen species.

The rapid induction of MT1 and MT2 upon stress and inflammation has designated these proteins as stress or acute phase proteins and this induction is, as for other acute phase proteins, regulated by stress hormones and inflammatory cytokines. Many groups have focused on elucidating the role of MT1 and MT2 in inflammatory diseases but unfortunately, reports do not always align. A key role for MT1/2 as (anti-)inflammatory mediators has frequently been proposed but has not been clarified to date. The most suitable example of this concept is the impact of MT1/2 on the LPS response. Following LPS injection in mice, MT1 and MT2 are induced in the liver and detectable in the plasma even before any other acute phase protein which justifies its nomination as acute phase or stress protein. However, while an increased responsiveness of MT-KO mice towards LPS is suggested, only one report effectively found increased liver damage following LPS in MT-KO mice [214–216]. In addition, the intact acute phase response in MT-KO mice further contests its functional elementary role as acute phase protein in this inflammatory model.

1.3.4. Metallothioneins as immune modifiers

The group of Lynes has focused on the immune-modulating properties of MT1/2 which clearly encompass both humoral and cellular immunity, as well as innate and adaptive immunological responses. They reported a suppressive effect of MT1/2 on humoral immunity. Mice immunized in the presence of exogenous MT developed a significant lower IgG response towards antigen provocation, which could be abolished by administration of MT1/2-specific monoclonal antibodies [217,218]. Complementary, a significant higher anti-ovalbumin response was observed in MT-KO mice [219].

Metallothioneins' effect on cellular immunity is more versatile. Recombinant MT1+2 induces the proliferation of cytotoxic lymphocytes [220,221], but suppresses the potential of macrophages to stimulate T-cell proliferation [222]. Metallothioneins stimulate lymphocyte proliferation but have a mitigating impact on their differentiation to and activity of cytotoxic T-lymphocytes [223]. On the other hand, MT1+2 induce the differentiation of naïve CD4+ T cells into IL-10 and transforming growth factor beta producing T cells and mice overexpressing *Mt1* showed reduced susceptibility and enhanced IL-10 level in collagen-induced arthritis [224]. Metallothionein 1 was recently found at the cell surface of DCs following $ZnCl_2$ treatment and these DCs induced the proliferation of regulatory T cells. Anti-MT antibodies targeting MT1 and MT2 were able to counteract the tolerogenic effect of $ZnCl_2$-treated DCs indicating that membrane-bound MT1 is essential for $ZnCl_2$-treated DCs to induce regulatory T cell proliferation. However, MT1 was not transported to the cell surface and had no effect on tolerogenic DC function induced by other stimuli than $ZnCl_2$ [225].

The MT1 and MT2 genes cluster nearby chemokine genes, which is located near an important IBD locus [69,226], and MT1/2 also exhibit chemokine-properties: they have the ability to drive the migration of leukocytes in a chemotactic gradient [226]. Chemokines are important regulators of leukocyte trafficking to damaged tissue, for example during inflammation. The appropriate influx of inflammatory cells results in efficient removal of tissue debris, necessary for rehabilitation. However, excessive infiltration of leukocytes during chronic immune stimulation may contribute to even more tissue damage and maintenance of inflammation. Metallothioneins are released upon stress and have been found at sites of inflammation [227–231]. The involvement of MT1/2 in the process of leukocyte

attraction and immune cell activation during intestinal inflammation, was part of this project.

2. Metallothioneins and inflammation

2.1. Modulation of MT1 and MT2 in non-IBD inflammatory models

The role of MT1/2 has been investigated in different inflammatory conditions using mice lacking functional *Mt1* and *Mt2* genes (MT-KO mice) or mice overexpressing *Mt1*. Most reports describe an increased sensitivity of MT-KO mice to experimental induced disease and a contribution of MT1/2 to protective mechanisms. For example, MT-KO mice are more susceptible to LPS-induced acute lung injury, to ovalbumin induced allergic airway inflammation, to oxidative lung injury and to LPS/D-GalN induced, oxidative and toxic acute liver injury [229,232–239]. Most reports attribute the increased sensitivity of MT-KO mice to reduced anti-oxidative mechanisms or disturbance in zinc distribution. However, overexpression of *Mt1* or zinc treatment does not always confer protection in the above mentioned conditions, indicating that other contributing factors may be important as well. Overexpression of *Mt1* does protect in some conditions in which excessive ROS production is involved such as (hypoxia/reperfusion-induced) injury in pancreatic beta cells and cardiomyocytes [203,204,240–242]. Contrarily, MT-KO mice were less affected during TNF-induced lethal shock and hyperoxic acute lung injury [243,244]. Thus, deletion of *Mt1* and *Mt2* or overexpression of *Mt1* yields different results in different conditions which are driven by distinct pathogenic mechanisms. Additionally, the (dis)advantage of MT1/2 might be time dependent in disease pathogenesis where different mechanisms dominate onset, progression and recovery of disease. This is nicely demonstrated in a number of experimental models associated with inflammation and oxidative stress. For example, in a mouse model for Alzheimer's disease, *Mt1*, *Mt2* and *Mt3* are detrimental at an early stage of disease but reduce detrimental effects at advanced age [245–247]. The MT-KO mice show increased numbers of apoptotic cells in skin transplants following UV-radiation but showed less epidermal hyperplasia during the epithelial regeneration phase compared to wild type controls [248,249]. Intriguingly, both MT-KO mice as well as mice overexpressing *Mt1* showed improved host defence against Listeria monocytogeneses infection compared to wild type

controls and this effect disappeared 3 days post inoculation [250]. The latter suggests that different mechanisms regulate disease progression in mice lacking and overexpressing *Mt1* and that the outcome most likely depends on balances of these regulating mechanisms.

2.2. Modulation of MT1 and MT2 in IBD models

The interest in MT1/2 as potential regulators of intestinal inflammation has two principal causes: (1) MT1/2 are considered as important anti-oxidantia and oxidative damage is involved in IBD pathogenesis and (2) MT1/2 function as zinc chaperones and zinc deficiencies are frequently observed during intestinal inflammation. Unfortunately, the results of reports exploring the role of MT1/2 in intestinal inflammation are inconsistent. There are two different MT-KO strains and both strains have been tested in DSS-induced colitis [191,192]. The two strains are generated by two different groups using a different targeted vector. As a result, the mice from Palmiter's lab still generate both MT1 and MT2 mRNA, but premature stop codons result in a 12 amino acid original MT2 sequence and a 10 amino acid MT1 sequence. In contrast, the mice from Choo's lab express only MT1 mRNA, which results in a 10 amino acid MT1 protein; MT2 is not transcribed due to interference in the transcription start site (*Table 2*).

Table 2. The mRNA and protein expression in MT-KO mice from Palmiter's and Choo's lab

	Palmiter		Choo	
	MT1	MT2	MT1	MT2
mRNA	yes	yes	yes	no
protein	10 AA	15 AA (12 MT specific)	10 AA	0 AA
Neo cassette	between MT1 and MT2		in MT2	

Additionally, both groups introduced the targeting vector into different mice strains. Palmiter's lab incorporated the construct into AB1 embryonic stem cells, positive clones were injected into C57BL/6 blastocytes and transplanted into females. Chimeric animals were crossed with 129/SvCPJ mice to obtain heterozygous mice (which were further mated to generate mice homozygous for Mt1 and Mt2 disruption). The mice originally produced by Palmiter's lab are commercially available at The Jackson Laboratory (Bar Harbor, Maine 04609 USA) and were used in the experiments described in chapter 2 and 3 of this thesis.

Choo's lab introduced the vector into the E14 embryonic stem cell line and positive clones were injected into blastocytes to create chimeric animals. Chimerics were mated to C57BL/6J mice to produce heterozygous animals. Thus, both strains are generated and maintained on a different background. Moreover, they have been backcrossed to other strains by several research groups which might additionally account for the differences in experimental outcome among groups.

Oz and colleagues used the MT-KO mice, produced by Masters et al., on the OLA129/SvCPJ background and reported no difference in DSS susceptibility between MT-KO mice, mice overexpressing *Mt1* nor wild type mice [251]. However, the mice overexpressing *Mt1* were on the C57BL/6J background and results of different groups can only be compared when they share the same genetic background. Tran and co-workers used the other MT-KO strain, produced by Michalska and Choo, on the OLA129/C57BL6/J background and backcrossed to C57BL/6J. Additional to the susceptibility of MT-KO mice to DSS-induced colitis, they also assessed the influence of zinc supplementation in this experimental setting. The MT-KO mice showed less myeloperoxidase activity, which is a measure for neutrophil infiltration, with a tendency towards a lower disease activity. Zinc supplementation during DSS colitis was able to lower the disease activity in wild type mice, no effect was observed in the MT-KO group [252]. The latest report exploiting MT knockout mice in colitis was published by Tsuji and colleagues who used the same strain as Tran. Contrarily, they reported an exacerbation of acute DSS-colitis in MT-KO mice [253]. Differences in outcome of experimental groups may be attributed to mouse strain, background, origin and designation, microbial environment and other experimental settings, which is further discussed in chapter 2 of this thesis.

Recently, MT1/2 were identified as critical factor for enhanced bacterial killing upon chronic PRR stimulation in human intestinal and mouse monocyte-derived macrophages. Metallothioneins were up-regulated in chronic NOD2-stimulated macrophages, which was regulated by NF-κB and caspase-1, and induced zinc-mediated autophagy [254]. The enhanced bacterial clearance upon chronic PRR stimulation is associated with decreased pro-inflammatory cytokine secretion and is assumed to be a protective mechanism to suppress tissue damage. The involvement of MT1/2 in this process might implicate important therapeutic potential since CD patients with NOD2 variants do not show enhanced bacterial killing nor reduced cytokine production following chronic NOD2 stimulation [66,255–258].

2.3. Expression of MT1 and MT2 in human intestinal inflammation

Different groups have reported on the expression of MT1/2 in IBD samples, however without uniformity. Lawrance and co-workers performed a microarray analysis to target differential mRNA expression in inflamed colonic specimens of UC and CD patients and found that MT1G and MT1H isoforms were decreased only in UC samples. Dooley et al. also performed a microarray analysis and found an up-regulation of MT1F, MT1G, MT1H in CD samples. Groups that addressed MT1/2 protein levels also reported conflicting results. The first studies used radio-immunoassay and silver-heme saturation on tissue homogenates and reported a decrease in total MT content [259,260]. Ioachim and Kruideniers showed a decreased epithelial MT immunoreactivity on immunohistochemical stained colonic tissue of IBD patients. Both studies did however not report on MT expression in the inflammatory infiltrate [261,262]. Contrarily, Bruwer et al. described an increased MT immunoreactivity, which was observed both in the intestinal epithelium and the infiltrate of IBD patients [263]. Differences in patient characteristics, sample handling and the detection methods that were used, may account for the discrepant results reported in literature. Our group has tried to clarify MT1/2 expression in IBD patients by comparing MT1/2 immunoreactivity in biopsies of IBD, infectious colitis and healthy control patients, which is discussed in chapter 2 of this thesis.

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AIMS – Elucidating the role of metallothioneins in IBD

The general aim of this work was to clarify the role of MT1/2 in intestinal inflammation and to explore the therapeutic potential of exploiting MT1/2 as treatment strategy for IBD patients. This thesis is divided in three chapters, each describing a particular subject of how MT1/2 might be integrated in the intestinal inflammatory response.

I. The relation between metallothioneins and the hypoxic adaptive response

Hypoxia is one of the main factors involved in the pathogenesis of IBD. A positive correlation between MT1/2 and hypoxia has been described for other inflammatory diseases. In **chapter 1**, we investigated whether MTs and HIF-1α, the key-player in the hypoxic pathway, are also interrelated in intestinal epithelial cells and in a mouse model for IBD.

II. The role of metallothioneins in the pathogenesis of murine colitis

To investigate the role of MT1/2 in intestinal inflammation, we used mice with the genetic deletion of *Mt1* and *Mt2* and a monoclonal anti-MT1/2 antibody in different mouse models for colitis. This work is described in **chapter 2**.

III. Impact of metallothioneins on macrophage phenotype and polarization

In chapter 2, we found that colitis is characterized by an infiltration of MT1/2 positive inflammatory cells, including macrophages. Macrophages are key players in the onset, progression and restoration of intestinal inflammation. We therefore explored the effect of MT1/2 on macrophage phenotype and polarization by using bone-marrow derived macrophages from wild type and MT1/2 knockout mice. This work is described in **chapter 3**.

Chapter 1

Metallothioneins and the Hypoxic Adaptive Response

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Inverse correlation between metallothioneins and hypoxia-inducible factor 1 alpha in colonocytes and experimental colitis

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Abstract

A positive-feedback mechanism between hypoxia-inducible factor 1 alpha (HIF-1α) and metallothioneins (MTs) has been identified in different diseases. Both proteins have been independently proposed in the pathogenesis of inflammatory bowel disease (IBD); however, their relation has never been studied in the gut. In this study, we investigated the interaction between HIF-1α and MTs in colonic epithelial cells and during experimental colitis. Dimethyloxalylglycine (DMOG) was used to subject colonocytes to hydroxylase inhibition and HIF-1α stabilization in three experimental models (*in vitro*, *in vivo* and *ex vivo*). Small interfering RNA targeting HIF-1α (siRNA-HIF) and MT (siRNA-MT) together with zinc-mediated MT induction were used to study the interaction between HIF-1α and MT in HT29 cells. Acute colitis was induced in C57BL/6 mice using dextran suphate sodium. MT expression and HIF-1α protein levels were measured using quantitative real-time PCR and ELISA respectively. Vascular endothelial growth factor (VEGF) expression was quantified as an indirect measure of HIF-1 transcriptional activity. DMOG down-regulated MT expression in HT29 cells, in freshly isolated human colonocytes and in colonocytes isolated from mice treated with DMOG ($p < 0.05$). SiRNA-HIF-treated cells displayed significant higher basal MT levels ($p < 0.05$) and an attenuated MT down-regulation after DMOG treatment. In turn, HIF-1α stabilization was significantly lower in zinc-treated control cells, displaying high MT levels, compared to siRNA-MT cells treated with DMOG ($p < 0.05$). In the course of experimental colitis, MT and VEGF mRNA expression were inversely related. MTs were induced in the acute phase and down-regulated during recovery. Opposing results were observed for VEGF expression levels ($p < 0.05$). The present study underscores the inverse relation between HIF-1α and MT expression in colonocytes and during experimental colitis. The manipulation of MTs may represent a novel therapeutic strategy for patients suffering from IBD.

Keywords**:** dimethyloxalylglycine; experimental colitis; hypoxia-inducible factor; hypoxic response, inflammatory bowel disease; metallothionein

1. Introduction

Metallothioneins (MTs) are low-molecular-weight, cysteine-rich proteins with pluripotent capacities in a variety of processes, including immunoregulation and apoptosis. More than 18 MT isoforms have been identified in mammalian cells, and they have been subdivided into the groups MT1 through MT4. While MT3 and MT4 are constitutively expressed, MT1 and MT2 are highly inducible. Their expression patterns and potential contributions to pathogenesis have been investigated in a broad range of pathological conditions [1,2]. Despite the established role of MTs in different pathological disorders, their regulation remains unclear and appears to be tissue-dependent.

Recently, a role for MTs in the adaptive hypoxic response has been proposed. An up-regulation of MTs under low oxygen tension was first observed in cancer cells [3]. Hypoxia-induced MT expression has been suggested to play a protective role in various tissues [4-8]. Additionally, MT over-expression or treatment with purified MT has been shown to protect against hypoxic damage in different cell types [8-13].

A direct interaction between MT and hypoxia-inducible factor 1 alpha (HIF-1α) has been demonstrated. Under normoxic conditions, prolyl hydroxylases (PHDs) hydroxylate HIF-1α, which leads to its degradation. However, PHDs are inhibited under low oxygen tension, which results in stabilization of HIF-1α. The stabilization of functional HIF-1α leads to transactivation of different target genes, such as vascular endothelial growth factor (VEGF), and is considered to be a cellular rescue mechanism from stress [14]. An increased HIF-1α transcriptional activity by MT over-expression in cardiomyocytes under hypoxic conditions has been reported [15] and MTs have been implicated in the protection of the kidney against chronic hypoxia through HIF-1α stabilization [8]. Taken together, these reports have revealed the up-regulation of MTs during hypoxia, which protects against hypoxic damage through oxygen radical scavenging and/or HIF-1α stabilization.

The expression and role of both MTs and HIF-1α have been independently investigated in inflammatory bowel diseases (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC). An aberrant MT expression pattern has been found in IBD. We and other investigators have observed the down-regulation of MTs in gut epithelium where HIF-1α appears to be

over-expressed in IBD patients and displays protective properties in murine models of intestinal inflammation [2, 16-18].

In this study, we examined whether a cooperative interaction between MTs and the hypoxic response could explain the observed MT down-regulation in IBD patients. The gut epithelium is located within a specific oxygen gradient and exists in a steady state of "physiological" hypoxia [19]. During inflammation, oxygen supply is insufficient, which is partly due to vasculitis and increased oxygen consumption by the inflammatory infiltrate. This low oxygen tension results in HIF-1α stabilization and activation of the hypoxic adaptive response. To investigate whether HIF-1α is able to control the transcriptional activation of MT isoforms in intestinal epithelial cells, colonocytes were treated with the hypoxia-mimicking pan-PHD inhibitor dimethyloxalylglycine (DMOG) to induce hydroxylase inhibition and HIF-1α stabilization in three experimental models. We revealed a HIF-1α-mediated inhibition of MT expression and investigated whether MTs are able to alter HIF-1α stabilization which may reinforce the effect of the hypoxic adaptive response, profoundly present during intestinal inflammation. Finally, we extrapolated our results to a model resembling ulcerative colitis.

2. Materials and methods

2.1. Cell culture

HT29 cells (HTB-38, ATCC Cell Biology Collection, Virginia, USA) were cultured in McCoy's5A medium supplemented with 10% foetal bovine serum and penicillin/streptomycin (Invitrogen, Merelbeke, Belgium).

2.2. Induction of hypoxia

HT29 cells were seeded at a density of 25 x 10^4 cells/well in a 24-well plate and exposed to atmospheric or 1% oxygen (O_2) in a hypoxic chamber (Oxoid, Hampshire, UK) the next day. After overnight incubation, cells were lysed in either RNeasy Lysis Buffer for RNA extraction or in radioimmunoprecipitation assay buffer for protein assessment.

2.3. DMOG treatment of colonic epithelial cells

In vitro treatment

HT29 cells were seeded at a density of 25 x 10^4 cells/well in a 24-well plate and treated with 1 mM DMOG (Bio-Connect, Huissen, the Netherlands) or vehicle (PBS) the next day. After 20 hours, cells were lysed for RNA extraction or protein assessment.

Ex vivo treatment

Eight human colonic biopsies from a healthy individual were collected during a colonoscopy and placed in RPMI medium containing a high dose of antibiotics (200 µg/ml gentamycin and penicillin/streptomycin from Invitrogen) supplemented with 10% foetal bovine serum. The biopsies were rinsed 3 times and incubated for 2 hours in medium at 37°C. Next, the biopsies were incubated overnight in RPMI medium containing 100 µg/ml gentamycin and 1 mM DMOG or PBS. Colonocytes were isolated the next day by incubating the biopsies 2 times for 30 minutes in medium containing 1 mM EDTA. After incubation, the biopsies were shaken gently to separate the epithelium. The remaining fragments were removed, and the resulting cell suspension was centrifuged and washed 2 times in PBS. The epithelial cells were subsequently lysed and used for RNA extraction. This study was approved by the local ethics committee (EC2000/242), and the volunteers signed and dated an informed consent form.

In vivo treatment

Eight-week-old SV129 mice were treated twice on alternating days with 8 mg DMOG (8 mg in 0.5 ml of sterile, endotoxin-free PBS) or PBS, intraperitoneally; this method was previously demonstrated to be effective for HIF-1α stabilization *in vivo* [20]. The mice were sacrificed the day after the second treatment by cervical dislocation. Colonic epithelial cells were isolated using Cell Recovery Solution (BD Bioscience, Belford, MA) and lysed for RNA and protein extraction [21]. The mice were treated according to institutional animal health care guidelines, following study approval by the Institutional Review Board of the Faculty of Medicine and Health Science of Ghent University.

2.4. Small interfering RNA

To knock down HIF-1α and MT levels, small interfering RNA (siRNA) was used (Supplementary materials and methods).

2.5. In vitro zinc treatment

The siRNA-MT and HT29 control cells were seeded at a density of 25 x 10^4 cells/well in a 24-well plate and treated the following day with 200 µM zinc acetate (Sigma Aldrich, Missouri, USA). Six hours after zinc treatment, 1 mM DMOG or an equivalent volume of PBS was added to the medium. Cells were lysed the next day for RNA and protein isolation.

2.6. In vitro treatment with N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN)

HT29 cells were seeded at a density of 25 x 10^4 cells/well in a 24-well plate and were treated the next day with 2.5 µM TPEN (Sigma Aldrich). Two hours after TPEN treatment, the cells were treated with 1 mM DMOG and were lysed 6 hours post TPEN treatment.

2.7. Experimental colitis

Thirty 8- to 10-week-old C57BL/6 mice received 4% dextran sulphate sodium (DSS, MP Biomedicals, Illkirch, France) in their drinking water for 7 days followed by 7 days of normal drinking water. The mice were matched for initial body weight prior to the start of the experiment, and their body weight was measured daily. Five animals from each group were anaesthetized with isoflurane in oxygen for blood sampling and were sacrificed by cervical dislocation at day (D) 0, D3, D7, D10 and D15. Serum samples were stored at -80°C. Colonic epithelial cells were isolated using the Cell Recovery Solution (BD), as previously described and were lysed for RNA and protein extraction [21]. Mice were treated according to institutional animal health care guidelines, following study approval by the Institutional Review Board of the Faculty of Medicine and Health Science of Ghent University (ECD 10/11).

2.8. RNA extraction

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Benelux, Venlo, the Netherlands) with on-column DNase treatment. Needle homogenisation was performed for epithelial cells isolated from mice and biopsies. The concentration and purity of the total RNA were determined using a spectrophotometer (WPA Biowave II, Isogen Life Science, the Netherlands).

2.9. quantitative real-time PCR

The sequences and qPCR efficiencies of all primer sets are listed in Supplementary Table 1.S. Quantitative real-time PCR protocol can be found in the Supplementary materials and methods.

2.10. HIF-1α ELISA

The HIF-1α protein content was measured by ELISA according to the manufacturer's protocol (human/mouse total HIF-1alpha DuoSet IC ELISA). The results were corrected for total protein content (Biorad Protein assay, California, USA).

2.11. Statistical analysis

Statistical analyses and figures were performed using GraphPad Prism® software (GraphPad Software, Inc., California, USA). Data are represented as the mean ± SEM. Differences between groups were compared using an unpaired t-test for normally distributed data and the Mann-Whitney U test for data that were not normally distributed. Correlations were analyzed using the Spearman's Rho test, and two-tailed probabilities were calculated. Results with p values < 0.05 were considered statistically significant.

3. Results

3.1. MTs are down-regulated in colonocytes after DMOG treatment

In an initial experiment using DMOG, we investigated the effect of hydroxylase inhibition on cultured colonic epithelial cells (HT29), on freshly isolated human colonocytes and on colonocytes isolated from *in vivo*-treated mice. The effectiveness of DMOG treatment was shown by HIF-1α protein stabilization in cultured colonocytes and transcriptional induction of VEGF in human and mouse colonocytes ($p < 0.05$; Supplementary Fig. 1.S.) [20].

All tested MT1 isoforms and MT2A were significantly under-expressed in DMOG-treated cultured cells ($p < 0.05$, Fig. 1A). Additionally, HT29 cells were exposed to 1% O_2 to complement the effect of DMOG, a chemical hypoxia mimicker, by overnight incubation in a hypoxic chamber. Twelve hours of exposure to 1% O_2 sufficiently down-regulated MTs expression in cultured colonocytes ($p < 0.05$; Fig. 1B).

Consistent with our *in vitro* results, primary colonocytes isolated from colonic biopsies treated with 1 mM DMOG showed a reduced expression of all tested MT1 isoforms and MT2A compared to colonocytes from PBS treated biopsies (Fig. 1C).

When analyzing the mRNA levels of the two Mt1 and Mt2 mouse isoforms, similar results were observed. The expression of Mt1 and Mt2 was significantly lower in colonocytes from *in vivo* DMOG-treated mice compared with vehicle-treated mice ($p < 0.05$; Fig. 1D).

3.2. MT down-regulation in colonic epithelial cells is mediated by HIF-1α

To determine whether the observed MT down-regulation after DMOG treatment is mediated by HIF-1α, HT29 cells were treated with siRNA-HIF before DMOG addition. HIF-1α was successfully down-regulated in siRNA-HIF-treated cells 48 hours post-transfection compared with siRNA-control-treated cells ($p < 0.001$, Supplementary Fig. 2.S.). The siRNA-HIF-treated cells displayed a significantly higher basal MT mRNA level compared with the siRNA-control cells ($p < 0.05$; Fig. 2A). MT down-regulation after DMOG treatment was attenuated in siRNA-HIF cells compared with control cells for the MT1A, MT1E, MT1F and MT2A isoforms (Fig. 2B).

Because zinc is essential to form functional HIF-1α, we confirmed HIF-regulated MT inhibition by pre-treating cultured HT29 cells with 2.5 µM TPEN, a known intracellular zinc chelator. We observed no change in VEGF mRNA induction after DMOG treatment in TPEN-treated cells (Fig. 2C). Moreover, no MT down-regulation could be observed in the TPEN-treated cells, confirming MT inhibition through functional HIF-1α (data shown for MT2A, $p < 0.05$; Fig. 2D). Noticeable, less MT2A down-regulation was observed after 4 hours of DMOG treatment in control cells (Fig. 2D) compared to cells subjected to 20 hours of DMOG (Fig. 1A).

3.3. MTs are able to suppress HIF-1α stabilization

Based on previous results demonstrating that MTs are able to stabilize HIF-1α in the kidney, we also investigated the role of MTs in HIF-1α stabilization in colonic epithelial cells [8]. We used siRNA to create HT29 cells that were deficient in MT expression (siRNA-MT). The siRNA-MT and control cells were first treated with zinc to induce MT synthesis to amplifying the effect. Subsequently, both groups were subjected to treatment with 1 mM DMOG. Significantly less MT up-regulation after zinc treatment was confirmed in the siRNA-MT group for MT1B ($p < 0.01$), MT1G ($p < 0.05$), MT1K ($p < 0.05$) and MT1X ($p < 0.01$) (Fig. 3A). Zinc-treated control cells, which displayed significantly higher MT levels, showed 6.4-fold less HIF-1α stabilization and significantly lower VEGF mRNA levels compared with siRNA-MT cells after DMOG treatment ($p < 0.05$, Fig. 3B and 3C).

3.4. MT and VEGF expression are inversely correlated in the course of colitis

In a final experiment, our aim was to extrapolate the previous results and elucidate the expression of MT and VEGF at different time points during acute colitis and recovery. Mouse colonocytes were isolated at different points, lysed and assessed for Mt1 and VEGF mRNA expression. The results demonstrated a negative correlation between both genes with an induction of Mt1 in the acute phase and a decrease in the recovery phase, while the opposite was true for VEGF (Fig. 4). A significant induction in Mt1 and a decrease in VEGF

expression were present at D7 and D10 ($p < 0.01$). Furthermore, Mt1 and VEGF showed an inverse correlation in each mouse during colitis ($R = -0.474$, $p < 0.05$).

4. Discussion

A role for MTs as mediators of inflammation has been implied in different inflammatory diseases. MTs are considered to be acute phase proteins because they are rapidly up-regulated during inflammatory processes and are able to attract leucocytes to the site of inflammation [23]. Their expression patterns and roles have been investigated in different pathological disorders and protective properties have been associated with high MT expression in models of inflammation [24]. Many groups have focused on the contribution of MTs in IBD; however, their regulation and role in intestinal inflammation remain unclear. We and other investigators have proposed that MTs are down-regulated during active IBD [2].

The intestinal epithelial lining forms a specific barrier in the gut and is in a steady state of ''physiological'' hypoxia. Adaptive mechanisms have evolved in the form of oxygen sensors which enable epithelial cells to respond to the limited oxygen supply [25]. Nevertheless, the intestinal epithelium displays a certain susceptibility to further decreases in oxygen supply, for example during inflammation. The sensitivity of intestinal epithelial cells to low oxygen tension and recent reports that have identified MT as hypoxia-inducible gene have led to the assumption of a correlation between MT and the hypoxic adaptive response in the intestinal epithelium and during intestinal inflammation.

In contrast to previous reports describing a positive-feedback mechanism between HIF-1α and MTs, we provide evidence for MT down-regulation through HIF-1α stabilization in three experimental models of colonic epithelial cells. The zinc chelator TPEN was used to confirm our results: no decrease in MT expression was observed without functional HIF-1α. Conversely, the attenuation of HIF-1α stabilization was observed in cells expressing high levels of MTs. While zinc chelation by TPEN treatment is able to inhibit the formation of functional HIF-1α, it is possible that the observed attenuation of VEGF induction is mediated by the capacity of MTs for zinc capitation and deprivation, which protects the cell from excessive metal toxicity [2,22]. This property could lead to less available zinc and,

subsequently, less functional HIF-1α stabilization after DMOG treatment in cells expressing high levels of MT.

This observed reciprocity between HIF-1α and MT was further explored during intestinal inflammation. MTs, which are considered acute phase proteins, were up-regulated during active inflammation and decreased during recovery in DSS-induced colitis. Additionally, the low VEGF expression in the acute phase and its up-regulation in the subacute and recovery phases are consistent with the manifestation of hypoxia during ongoing mucosal inflammation and the need for activation of the protective pathway to restore barrier function. Both findings are consistent with a reduced expression of MTs and up-regulation of HIF-1α in the gut epithelium of IBD patients suffering from chronic intestinal inflammation [2,16]. While HIF-1α protein levels have protective properties in murine models of intestinal inflammation [17,18,20,26], no beneficial or detrimental effects could be demonstrated for MT levels in two independent murine colitis models [27,28]. Both latter studies have described MT up-regulation using a DSS-induced model of colitis. However, these reports did not examine MT expression during the course of colitis. The regulation and role of MTs may be critically time-dependent during intestinal inflammation. Furthermore, *in vivo* models are required to evaluate whether the observed acute induction of MT or its decrease during recovery demonstrates an essential adaptive mechanism. While the over-expression of MTs leads to HIF-1α stabilization in kidney tubular cells and cardiomyocytes, our results distinguish the intestinal epithelium from other cell types by revealing an inverse correlation between both [8,15].

In conclusion, we demonstrated the HIF-1α-mediated down-regulation of MTs in colonic epithelial cells. MTs were able to attenuate HIF-1α stabilization possibly through zinc deprivation in HT29 colonic epithelial cells. The negative-feedback regulation of colonic epithelial MT expression by HIF-1α could suggest an intestinal rescue mechanism during the acute phase of inflammation as demonstrated by the time-dependent expression of both during experimental colitis. The previously observed low MT profile in IBD patients may point to a hypoxia-driven adaptive response during the course of gut inflammation to restore the intestinal epithelial barrier and reduce further damage by pro-inflammation-dependent mechanisms. Agents that target MTs and enhance the protective effects of HIF-1α may represent future therapeutic opportunities for IBD patients.

Disclosures/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure and table legends

Figure 1 Fold reduction in metallothionein (MT) mRNA expression in colonocytes after dimethyloxalylglycine (DMOG) treatment

(A) The mRNA expression of different MT isoforms was down-regulated in HT29 cells after 20 hours of DMOG treatment and (B) after overnight incubation in a hypoxic chamber under 1% O_2. The solid line represents a 4-fold (A) and a 1.5-fold (B) down-regulation (*n* = 4). (C) The mRNA expression of different MT isoforms was down-regulated in colonocytes isolated from DMOG-treated biopsies. The solid line represents a 2-fold down-regulation (*n* = 1, pool of 4 biopsies). (D) Mt mRNA expression is down-regulated in mouse colonocytes after *in vivo* DMOG treatment. The solid line represents a 2.5-fold down-regulation (*n* = 4). Fold reductions are shown. * $p < 0.05$; ** $p < 0.01$.

Figure 2 Hypoxia-inducible factor 1 alpha (HIF-1α) down-regulates metallothionein (MT) expression

Small interfering RNA (siRNA) targeting HIF-1α was used to evaluate the effect of HIF-1α on MT expression in HT29 cells. Cells were transfected with siRNA-HIF or siRNA-control and treated 48 hours post-transfection with 1 mM DMOG. Additionally, the zinc chelator TPEN was used to confirm the necessity of functional HIF-1α to down-regulate MTs. (A) Basal MT mRNA expression was significantly higher in siRNA-HIF-treated cells compared with siRNA-control-treated cells. Fold inductions are shown (*n* = 4). (B) MT down-regulation was attenuated in siRNA-HIF cells after DMOG treatment compared with control cells for MT2A, MT1A, MT1E, MT1F and MT1G. Fold reductions are shown (*n* = 3). (C) TPEN successfully inhibited the formation of functional HIF-1α as shown by attenuated vascular endothelial growth factor (VEGF) induction (*n* = 4). (D) No MT2A down-regulation after DMOG-treatment was observed in TPEN-treated HT29 cells compared with control cells (n = 4). * $p < 0.05$; *** $p < 0.001$.

Figure 3 Metallothioneins (MTs) are able to attenuate hypoxia-inducible factor 1 alpha (HIF-1α) stabilization

The siRNA-mediated silencing of MTs was used to evaluate the effect of MTs on HIF-1α stabilization in HT29 cells. Zinc was used to induce MT synthesis in siRNA-MT cells and control cells. Both zinc-treated, siRNA-MT and control cells were treated with 1 mM DMOG to evaluate HIF-1α stabilization and vascular endothelial growth factor (VEGF) induction in both groups. (A) The MT induction by zinc was attenuated in siRNA-MT cells for MT1B, MT1G, MT1K and MT1X, confirming the efficacy of siRNA-MT. Transcripts were normalized against the MT levels in siRNA-MT cells (n = 3). (B) The HIF-1α protein (n = 2) and (C) the VEGF mRNA levels (n = 3) were significantly less increased after DMOG treatment in zinc-stimulated HT29 cells compared with the stimulated siRNA-MT cells. * $p < 0.05$; ** $p < 0.01$.

Figure 4 Negative correlation between mouse metallothionein 1 (Mt1) and vascular endothelial growth factor (VEGF) expression in the course of colitis

Mice were treated with 4% dextran sulphate sodium (DSS) for 7 days followed by 7 days of normal drinking water. Colonocytes were isolated when mice were sacrificed at different time points (day (D) 0, D3, D7, D10 and D15) and the mRNA expression of Mt1 and VEGF was quantified using quantitative real time-PCR. The results were normalized to baseline levels (D0). A significant Mt1 induction and a decrease in VEGF expression were present at D7 and D10. Mt1 and VEGF were inversely correlated in each mouse during colitis (R = -0.474, $p < 0.05$). The data are presented as the mean ± SEM (n = 5 per group). ** $p < 0.01$.

Figure 1

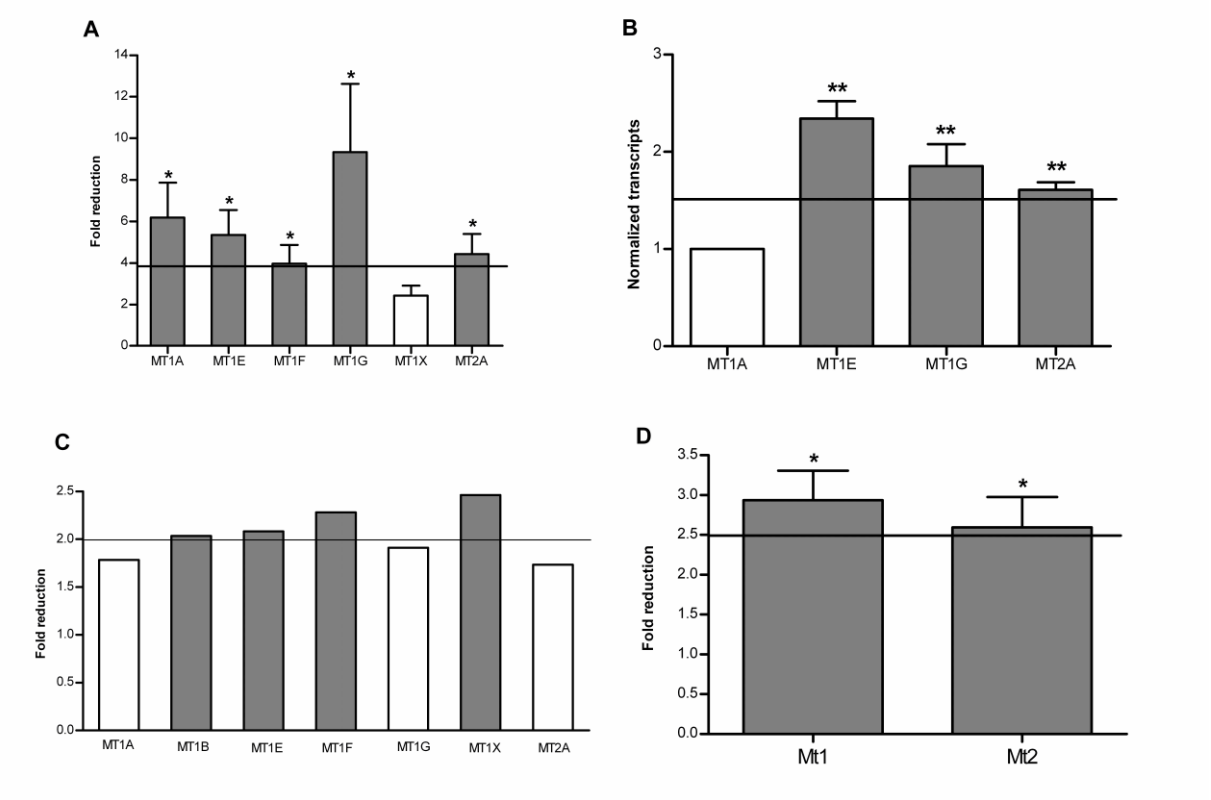


Figure 2

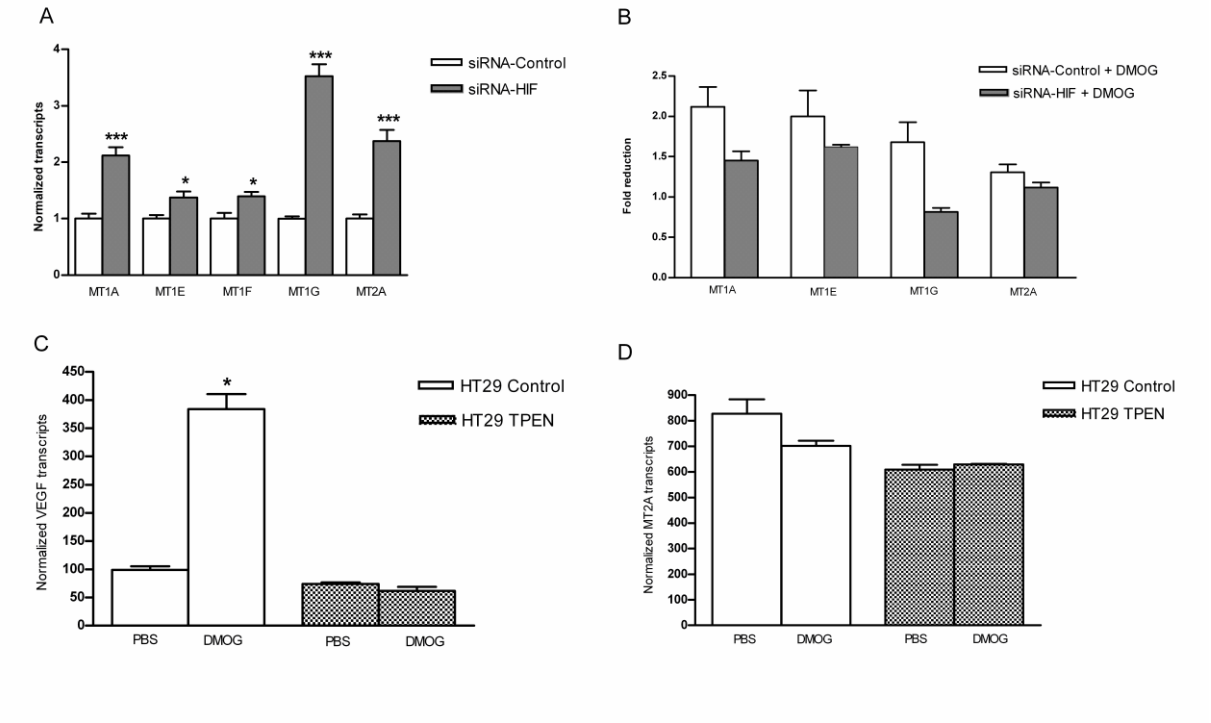


Figure 3

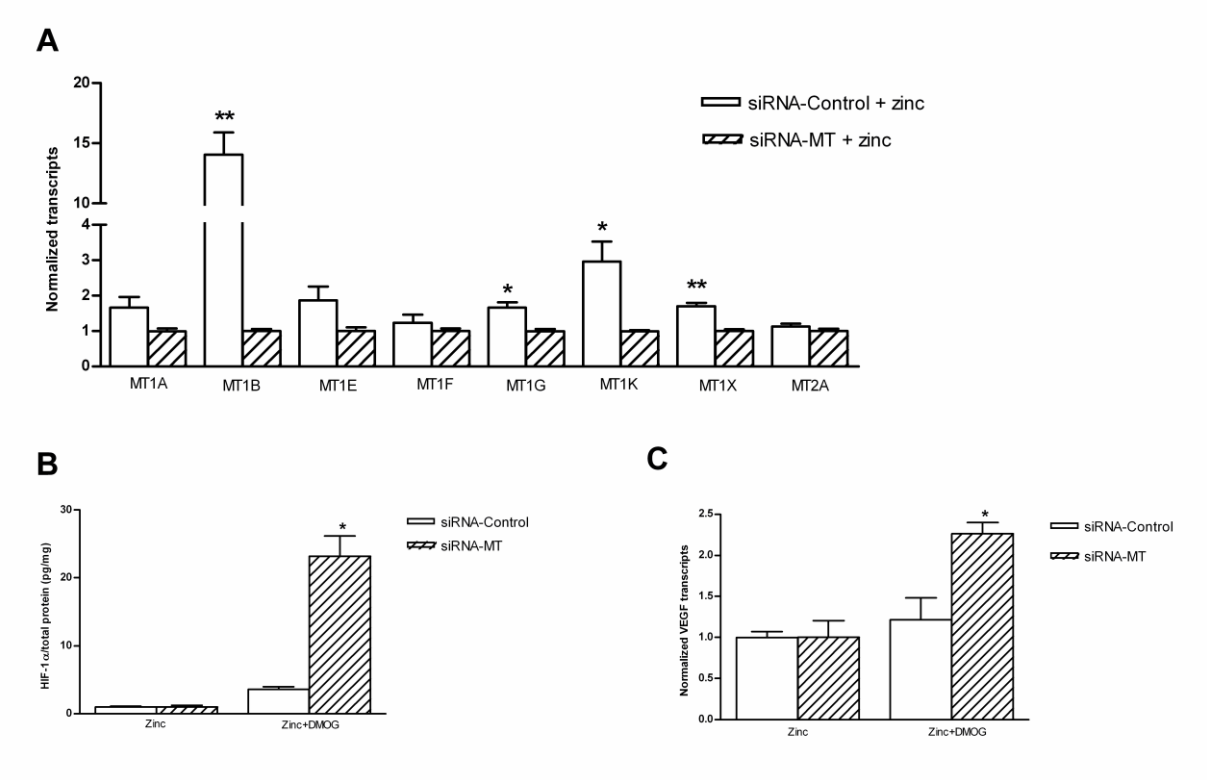
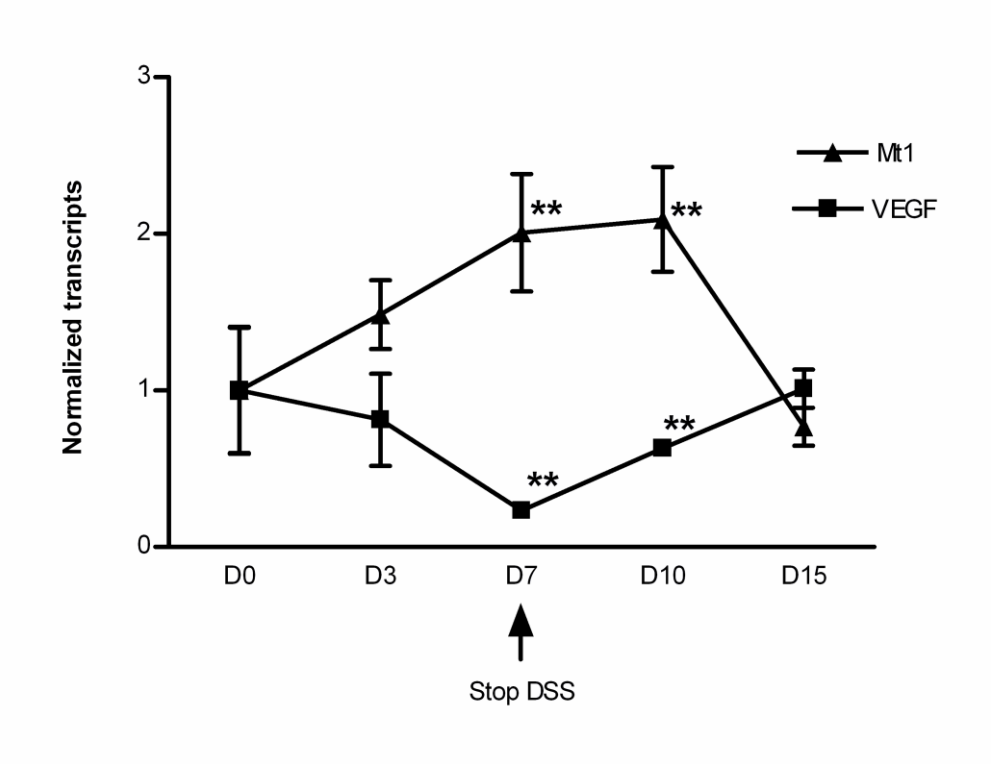


Figure 4



Supplementary data

1. Supplementary Materials and methods

1.1. Small interfering RNA

To knock down the HIF-1α levels, HT29 cells were seeded at a density of 25 x 10^4 cells/well in a 24-well plate and were transfected the next day with either small interfering RNA (siRNA) duplexes targeting human HIF-1α or control siRNA (Biolegio, Nijmegen, The Netherlands) using 5 µl of lipofectamine (Invitrogen) in 500 µl of RPMI medium [18]. The siRNA sequences for HIF-1α and the scrambled control sequence used for duplex formation were AGU UAG UUC AAA CUG AGU UAA UCC C and CAA GAC CCG CGC CGA GGU GAA TT, respectively. Forty-eight hours after transfection, cells were treated with either 1 mM DMOG or an equivalent volume of PBS and were lysed the next day.

To knock down MT levels, a stable MT siRNA expressing cell line was created (siRNA-MT). A short hairpin RNA fused to the H1 promoter was synthesized by PCR and placed in the pSUPER vector (Tronolab, Lausanne, Switzerland) using the primers 5'-CAA TCT CTT GAA **TTG CAC TTG CAG GAG CCG G**GG GGA TCT GTG GTC TCA TAC AGA ACT TAT AA-3' and 5'-CCA TCG ATT TCC AAA AA**C CGG CTC CTG CAA GTG CAA** TCT CTT GAA TTG C-3' (scrambled control). The sequence in bold is a 19-mer that specifically targets MT and is present in MT1B, MT1E, MT1H, MT1J and MT1M. A siRNA control cell line was created with the primers 5'-CAA TCT CTT GAA **TTG CAC TCT TTG CAC TTG C**GG GGA TCT GTG GTC TCA TAC AGA ACT TAT AA-3' and 5'-CCA TCG ATT TCC AAA AA**G CAA GTG CAA AGA GTG CAA** TCT CTT GAA TTG C-3'. The PCR fragment was cloned into the pLVTH-siGFP vector (Tronolab). To produce virus for short hairpin RNA delivery, HEK293T cells (ATCC CRL1573) were transfected using the calcium phosphate method with 3 µg of pCMV-d8.91 (Tronolab), 1.5 µg of PMDG2 (Tronolab) and 1.5 µg of the shRNA construct. After 48 hours, the supernatant containing the viral particles was harvested and passed through a 0.45 µm filter. One day before viral transduction, HT29 cells were seeded at a density of 50 x 10^3 cells/well in a 24-well plate. The cells were overlaid with viral supernatant and were centrifuged for 1 hour at 32°C. This procedure also was repeated with the 72-hour viral supernatant. Cells expressing high levels of GFP were subsequently

sorted with an EPICS Altra cell sorter (Beckman Coulter, Woerden, The Netherlands).

1.2. quantitative real-time PCR

One microgram of total RNA was converted to single strand complementary DNA (cDNA) by reverse transcription (Superscript, Invitrogen) with oligo(dT) priming. The cDNA was diluted 1/8 and was used in real-time quantification with SYBR Green (Roche, Vilvoorde, Belgium) and 250 mM of each primer. A two-step program was run on a LightCycler® 480 (Roche). Cycling conditions were 95°C for 10 minutes and 45 cycles of 95°C for 10 seconds and 60°C for 1 minute. A melting curve analysis confirmed primer specificity. All reactions were performed in duplicate and normalised to glyceraldehyde phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex subunit A (SDHA) (for cultured HT29 cells and human biopsies) or to hydroxymethylbilane synthase (HMBS) (for mouse epithelial cells). The PCR efficiency of each primer pair was calculated using a standard curve from reference cDNA. The amplification efficiency was determined using the formula $10^{-1/slope}$.

2. Supplementary Figure legends

Figure 1.S. Dimethyloxalylglycine (DMOG) induces hypoxia-inducible factor 1 alpha (HIF-1α) protein stabilization and vascular endothelial growth factor (VEGF) RNA levels

HIF-1α protein levels and RNA VEGF levels are significant increased after DMOG treatment in HT29 cells (A). RNA VEGF levels are significant increased in mice colonocytes after in vivo DMOG treatment (B). RNA VEGF levels are increased in human colonocytes after biopsy treatment with DMOG (**C**). * $p < 0.05$.

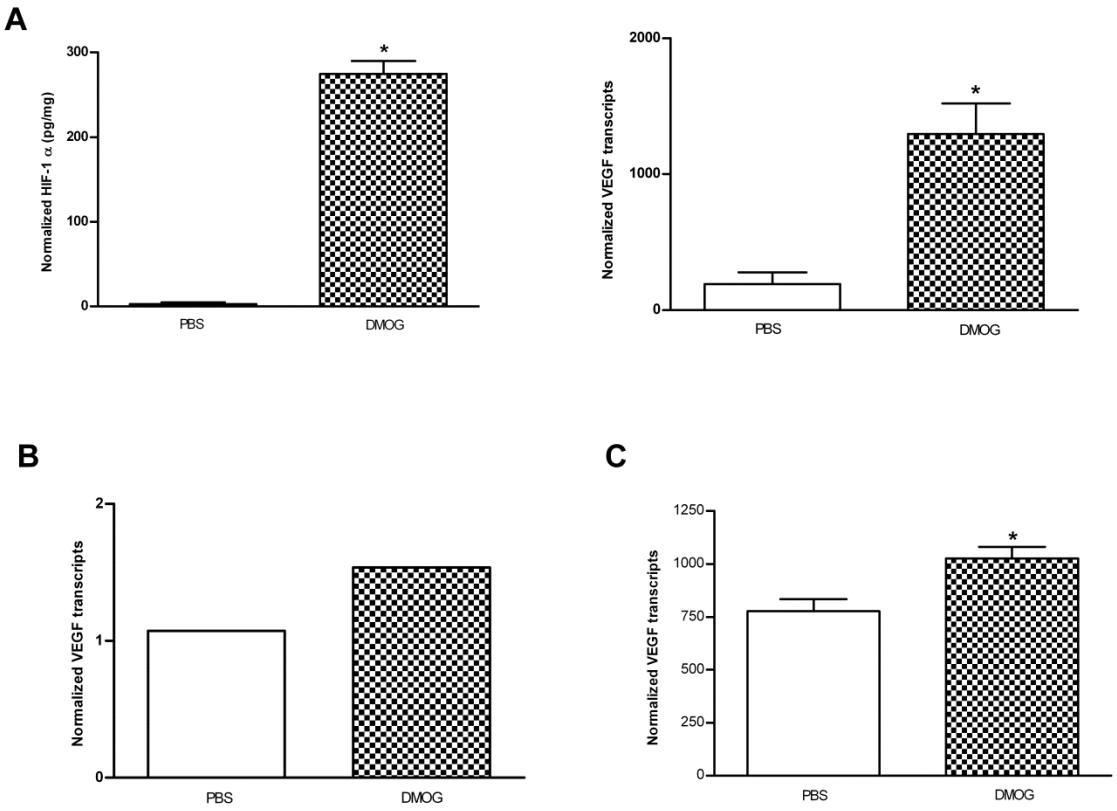
Figure 2.S. SiRNA targeting HIF was successful 48 hours post transfection

HIF-1α protein levels were significantly lower in siRNA-HIF-treated cells compared to siRNA-control-treated cells (n = 2) (A). HIF-1α protein stabilization (n = 2) (B) and vascular endothelial growth factor (VEGF) mRNA induction (n = 4) (**C**) were attenuated in siRNA-HIF cells compared with siRNA-control cells after DMOG treatment, confirming the siRNA efficacy.***p<0.001.

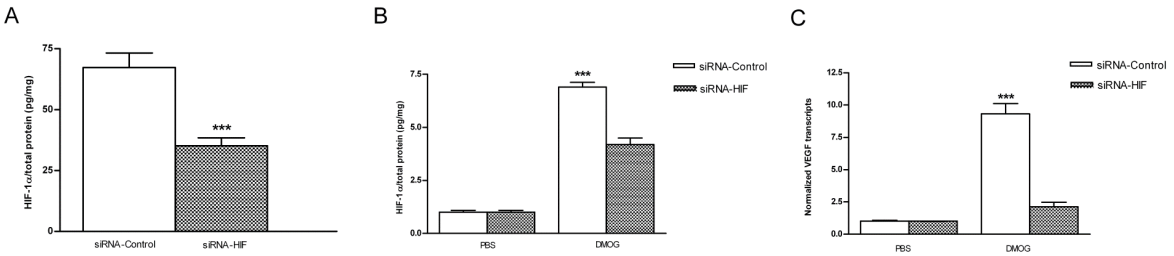
Table 1.S. Sequences and qPCR efficiencies of the primer sets used for quantitative real time-PCR in this study

Gene symbol	Reference sequence	Forward primer	Reverse primer	Efficiency	R^2
Human					
GAPDH	NM_002046	TGC ACC ACC AAC TGC TTA GC	GGC ATG GAC TGT GGT CAT GAG	91	0.9936
SDHA	NM_004168	TGG GAA CAA GAG GGC ATC TG	CCA CCA CTG CAT CAA ATT CAT G	92	0.9947
VEGF	NM_001025366	TCGGGAACCAGATCTCTCAC	TCTGTCGATGGTGATGGTGT	105	0.9508
MT1A	NM_005946	GCA AAG GGG CAT CAG AGA AGT G	AAA TAC AGT AAA TGG GTC AGG GTT G	92	0.9768
MT1B	NM_005947	AAG TGC TGC TGC TCT TGC TG	TGG TTG CTC TAT TTA TGT CTG GGA G	98	0.989
MT1E	NM_175617	TCA GGT TGG GAG GGA ACT CAA	GAA AGC CTG GAG AGG GAA TGA	99	0.9974
MT1F	NM_005949	GCG ACT GAT GCC AGG ACA AC	CAC AGG AAA AGG AAT GTA GCA AAT G	95	0.9953
MT1G	NM_005950	ACAGCCCTGCTCCCAAGTA	GGAATGTAGCAAAGGGGTCA	103	0.9798
MT1K	NM_176870	CTG CAA AGG GAC GTT GGA GAA C	CAG CAA ATG GCT CAG TAT CGT ATT	92	0.9896
MT1X	NM_005952	GCAAATGCAAAGAGTGCAAA	GCACTTGTCTGACGTCCCTT	94	0.9991
MT2A	NM_005953	AAA GGG GCG TCG GAC AAG TG	GAA TAT AGC AAA CGG TCA CGG TCA G	95	0.9745
Mouse					
HMBS	NM_013551	AAG GGC TTT TCT GAG GCA CC	AGT TGC CCA TCT TTC ATC ACT G	95	0.9944
VEGF	NM_001025250	ACTCGGATGCCGACACGGGA	CCTGGCCTTGCTTGCTCCCC	98	0.998
Mt1	NM_013602	GCTGCTCCTGCTGTCCCGTG	GGTGGCAGCGCTGTTCGTCA	92	0,9988
Mt2	NM_008630	CAGCCCTGGGAGCACTTCGC	TCGCCATGGACCCCAACTGC	100	0,9996

Supplementary figure 1



Supplementary figure 2



Corrigendum to references

Reference 21 “B. Rana, D. Mischoulon, Y. Xie, N.L. Bucher, S.R. Farmer, Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes: reciprocal expression of C/EBP alpha and immediate-early growth response transcription factors, Mol. Cell. Biol. (1994) 5858-5869” should be replaced by the reference “N. Perreault and J. Beaulieu, Primary Cultures of Fully Differentiated and Pure Human Intestinal Epithelial Cells, Exp. Cell. Res. (1998) 34-42”.

Chapter 2

Metallothioneins and Intestinal Inflammation

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ORIGINAL PAPER

Role of metallothioneins as danger signals in the pathogenesis of colitis

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Role of metallothioneins as danger signals in the pathogenesis of colitis

Running title: Metallothionein inhibition suppresses murine colitis

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Conflict of interest: L Devisscher, M Lynes, M De Vos and D Laukens are listed as co-inventors on a patent application protecting the use of MT antagonists to treat intestinal inflammation (WO2013007678).

Word count: 3999

Abstract

Inflammatory bowel diseases (IBD) are recurrent intestinal pathologies characterized by a compromised epithelial barrier and exaggerated immune activation. Mediators that influence this chronic process of immune cell infiltration may represent new therapeutic opportunities. Metallothioneins (MTs) are stress-responsive proteins with immune-modulating functions. Metallothioneins have been linked to IBD, but their role in intestinal inflammation is inconclusive. We investigated MT immunoreactivity in colonic biopsies from IBD and acute infectious colitis patients and healthy controls and evaluated MT's role in experimental colitis using MT knockout mice and anti-MT antibodies. Antibody potential to target extracellular MT and its mechanism was tested *in vitro*. Biopsies of patients with active colitis showed infiltration of MT positive cells, in a pattern that correlated with the grade of inflammation. MT knockout mice displayed less severe acute dextran sulfate sodium (DSS)-induced colitis compared to congenic wild type mice based on survival, weight loss, colon length, histologic inflammation and leukocyte infiltration. Chronic DSS-colitis confirmed that *Mt1* and *Mt2* gene disruption enhances clinical outcome. Blockade of extracellular MT with antibodies reduced F4/80 positive macrophage infiltration in DSS- and trinitrobenzene sulfonic acid-colitis with a tendency towards a better outcome. Whole-body single-photon emission computer tomography of mice injected with radioactive anti-MT antibodies showed antibody accumulation in the colon during colitis and clearance during recovery. Necrotic and not apoptotic cell death resulted in western blot MT detection in HT29 cell supernatant. In a Boyden chamber migration assay, leukocyte attraction towards necrotic cell supernatant could be abolished with anti-MT antibody, indicating chemotactic potential of endogenous released MT. Our results show that human colitis is associated with infiltration of MT positive inflammatory cells. Since antibody blockade of extracellular MT can reduce colitis in mice, MT may act as a danger signal and may represent a novel target for reducing leukocyte infiltration and inflammation in IBD patients.

Keywords: danger signal, inflammatory bowel diseases, metallothioneins, murine colitis

Introduction

Inflammatory bowel diseases (IBD) are chronic intestinal inflammatory diseases that can affect the entire gastrointestinal tract (Crohn's disease, CD) or remain restricted to the colon (ulcerative colitis, UC). A combination of genetic predisposition, gut microbiota and environmental factors are thought to contribute to excessive immune activation and disruption of the intestinal layer in IBD, however the exact etiology is still unclear. As a consequence, therapeutic options for IBD patients are limited [1-3].

Metallothioneins (MTs) are stress-responsive proteins that are induced by a variety of stimuli including infection and inflammation and may harbour anti-inflammatory properties by for example scavenging reactive oxygen species [4]. Metallothioneins have also been found in extracellular compartments at sites of inflammation and can modify a number of immune activities [5-7]. Metallothioneins have been implicated in the pathogenesis of several inflammatory diseases, including IBD. Attempts to characterize MT expression in human IBD have yielded contradictory results that may be the result of differences in sampling, processing, and detection methods [8]. Similarly, murine models that explored MT's role in intestinal inflammation have produced inconclusive results that may reflect differences in experimental design [9-11]. We, and others, have previously showed that MTs are up-regulated during murine colitis [11,12]. In this work, our aim is to clarify the consequence of MT expression in IBD, to focus on MT-mediated leukocyte trafficking and inflammation and to unravel MT's role in colitis using validated mouse models.

Materials and methods

Human samples

Colonic biopsies of 37 CD, 20 UC, 16 acute infectious colitis patients and 15 healthy controls were formalin fixed, paraffin embedded and evaluated for severity of colitis and MT immunoreactivity (see Table 1 for patients characteristics). Patients were diagnosed based on clinical, endoscopic and histologic criteria. Control biopsies were obtained from patients who underwent colonoscopy for follow up of polyp detection or colorectal carcinoma

screening. Colitis was histologically scored in blinded samples by two independent pathologists on hematoxylin and eosin (H&E)-stained sections using a modification of the grading scale described by Geboes *et al* [13]. Sections were classified as mild (1 or 2 crypt abscesses, small number of infiltrated granulocytes), moderate (multiple crypt abscesses, moderate infiltration of granulocytes) or severe (ulcers, large granulocytic infiltrate) (Table 1). MT staining was carried out with the NexES IHC automated staining system (Ventana Medical Systems, Tucson, AZ) using mouse monoclonal anti-MT clone E9 (Zymed Laboratories, San Francisco, CA). An isotype-specific irrelevant antibody (mouse IgG1, Dako, Glostrup, Denmark) was used in a matched concentration to control for non-specific binding of the primary antibody. Sections were analysed by two independent observers using a semi-quantitative grading system: 0: no MT-positive cells; 1: single MT-positive cells or foci (1 to 5%); 2: moderate number of foci of MT-positive cells (6 to 40%); 3: large number of MT-positive cells (40 to 70%); 4: majority of cells are MT-positive (>70%) and mean scoring values of the two observers were plotted against the severity of colitis. This study was approved by the local ethics committee of University Hospital Ghent (EC UZG 2000/242, 2004/242 and 2006/362) and involves Caucasian participants.

Mice

DSS-induced colitis was evaluated in wild type (WT) and MT knockout (MT-KO) congenic mouse strains sharing the C57BL/6J background. The mice were bred at the University of Connecticut from strains produced as previously described [14,15]. In studies to evaluate monoclonal anti-MT antibody therapy, WT C57BL/6 mice were purchased from Harlan (Boxmeer, The Netherlands). Animals were reared and housed in the laboratory animal facility at University Hospital Ghent according to the institutional animal healthcare guidelines. Mice from different experimental groups were co-housed during the experiment in order to homogenize gut microbiota between groups. This study was approved by the Institutional Review Board of the Faculty of Medicine and Health Science of Ghent University (ECD 10/11).

Colitis induction in WT and MT-KO mice

Acute colitis: Thirty 10-week-old mice per group received 4% dextran sulphate sodium (DSS, MW 36000-50000; MP Biomedicals, CA, US) in drinking water for 7 days followed by 7 days of normal drinking water. The mice were matched for body weight prior to start of the experiment. Five animals per group were sacrificed by cervical dislocation on day 10; the remainder of the animals were clinically monitored until day 15. Colon lengths were measured and distal colon samples were flash-frozen for assessment of myeloperoxidase (MPO) activity as previously described [16] and fixed in formalin for H&E and F4/80 immunostaining.

Chronic colitis: Sixteen 12-week-old mice per group received 3 cycles of 5 days of 4% DSS in drinking water, followed by 7 days of normal drinking water. The mice were analysed for survival and body weight loss and sacrificed at the end of the final cycle [17].

Antibody treatment during acute colitis

DSS-induced colitis: Twenty-four 10-week-old WT C57BL/6 mice received 4% DSS through drinking water for 7 days and were followed up for weight loss and survival until day 10. Day 10 was chosen as end point based on the previous DSS experiment showing maximum weight loss at this day. The mice were randomized on day 4 and treated intraperitoneally (i.p.) with 4 mg/kg monoclonal anti-MT antibody (clone UC1MT) or an equivalent dose of an irrelevant IgG (control) on days 4, 6 and 8 [6]. Colon samples were harvested as described in the previous section.

Trinitrobenzene sulfonic acid (TNBS)-induced colitis: Fifteen 10-week-old WT C57BL/6 mice were treated intrarectally with 100 µl of 2.5% TNBS in ethanol (EtOH) on day 0 [18]. Mice received daily i.p. injections of 4 mg/kg UC1MT antibody or IgG control. Four mice only received EtOH intrarectally as control for the TNBS/EtOH treatment. Samples were collected at day 3, as described for the DSS experiment.

Evaluation of murine colitis

Colon sections were H&E stained and blindly scored for inflammation by two observers using a validated scoring system [19].

Immunohistochemistry for F4/80

Paraffin-embedded mouse colon sections were rehydrated by serial immersion in xylene and ethanol and pre-treated with Antigen Retrieval solution (Dako, Glostrup, Denmark). After blocking with 3% peroxidase (in methanol) and rabbit serum (Dako), the primary antibody (1/200 rat anti-F4/80, Serotec, Dusseldorf, Germany) was applied overnight. Rabbit anti-rat Ig (in rabbit serum) was applied for 45 min, followed by the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, USA) and the chromogen 3,3'-diaminobenzidine (DAB) (Dako). Counterstaining was performed with hematoxylin. Computerized semi-quantitative analyses were performed using Cell D software (Olympus Imaging Solutions, Münster, Germany).

Small animal imaging of mice injected with anti-MT antibodies

Indium (^{111}In) labelling of anti-MT antibodies (clone UC1MT): antibody conjugation was performed under strict metal-free conditions using the bifunctional chelator p-SCN-Bz-DOTA (Macrocyclics, Dallas, TX). Antibodies were transferred in metal-free 0,1M NaHCO3 (pH 8.5) via Centricon YM-30 (Cenfrigual Filter Device, Millipore, Darmstadt, Germany) at 3 mg/ml. Chelator conjugation to the antibody was performed at a 20:1 concentration overnight (4°C). Buffer exchange into 0.1 M ammonium acetate (pH 6.5) and removal of unbound chelator was performed by Centricon YM-30 ultrafiltration. The pH was adjusted to 6.0 with metal-free ammonium acetate. Typically, labelling reactions were performed using 1 mg of antibody and 185 MBq of $^{111}InCl_3$ (in 0.05M HCl) (Covidien, Mansfield, MA). DOTA conjugates were incubated for 60 min at 37°C. The reaction was quenched by adding a 100-fold excess of Na2EDTA. Removal of free ^{111}In was performed by Centricon YM-30 ultrafiltration. Labelling yields and radiochemical purities were higher than 95%.

Imaging acquisitions: WT C57BL/6 mice received 4% DSS in drinking water, followed by 7 days of normal drinking water to induce acute colitis. On day 0 (control), 7 (acute colitis) and 14 (recovery) of DSS-colitis, mice (*n* = 4-6) received 35.0 ± 2.6 MBq ^{111}In-labeled UC1MT intravenously. Two days after tracer injection (ensuring proper blood clearance of the antibody through the liver and kidneys), 0.5 ml gastrografin was administered rectally followed by computed tomography (CT, at 50 kV and 612 µA) and whole-body single-photon emission CT (SPECT) using the pre-clinical U-SPECT-II/CT system (MILabs, Utrecht, the

Netherlands). The mice were anesthetized with isoflurane in oxygen and body temperature was maintained using a heated bed.

Data Analysis: SPECT acquisitions were iteratively reconstructed using ordered-subset expectation maximization [20]; 3 iterations were used in combination with 16 subsets and images were filtered using a Gaussian filter with a 1 mm full-width half-maximum. CT images were analytically reconstructed using the Feldkamp algorithm [21]. Volumes of interest (VOIs), determined on sagittal, coronal and transversal reconstructed CT slices, were drawn over the colon and entire mouse body using AMIDE [22]. Based on the global counts measured in the VOIs on the SPECT images, the percentage of injected dose per gram tissue (%ID/g) in the colon was calculated.

Autoradiography: colon sections from SPECT-analysed animals were rinsed, immersed in embedding matrix (Cryocompound, Klinipath, Olen, Belgium), cut into 20 µM slices on a cryostat, and mounted on glass slides. The sections were exposed to a detector screen in an autoradiography cassette (FBCS 810, Fisher Scientific, Aalst, Belgium). After 15 min, screens were imaged by the Cyclone Plus Storage Phosphor System (PerkingElmer, Zaventem, Belgium). Consecutive sections were stained with H&E and analysed for inflammation [19].

Cell culture

HT29 cells (HTB-38, ATCC Cell Biology Collection, Virginia, USA) were cultured in McCoy's 5A medium (Invitrogen, San Diego, CA, USA), supplemented with 10% FBS. Cells were seeded at 2.5×10^5 cells/well in 24-well plates and exposed for 6 and 24 h to 50 ng/ml tumor necrosis factor (TNF)-α (Invitrogen) + 300 ng/ml interferon (IFN), to 2 µM and 10 µM staurosporine (Sigma) + 300 ng/ml IFN and to 300 ng/ml IFN alone. IFN was added to these treatments since IFN co-signalling is required for TNF-induced cell death and enhances staurosporine-induced apoptosis in HT29 cells [23-25]. Nonspecific necrosis was induced by freeze/thaw cycles in liquid nitrogen-room temperature. All conditions were preceded by 250 µM zinc acetate (Sigma) treatment to up-regulate intracellular MT expression and amplify the effect. Cell supernatants were collected for MT detection by Western blot and lactate dehydrogenase (LDH) activity measurement (using an LDH Activity Assay Kit from Sigma). The activity of caspase-3/7 in cultured cells was analysed using the Caspase-Glo® 3/7 Glo Assay Kit (Promega, Leiden, the Netherlands) according to manufacturer's instructions.

Western blot analysis for MT

Twenty µl sample was mixed with 1:4 loading buffer (Invitrogen) and 1:10 reducing agent (Invitrogen). Samples were denatured, separated on 4–12% Bis-Tris SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare, Waukesha, Wisconsin, USA). Blotted membranes were incubated with 2.5% glutaraldehyde in distilled water for 1 h and in 50 mM monoethanolamine/PBS for 5 min. After blocking with 10% milk powder in PBS with 0.1% Triton-X100 (block buffer), blots were incubated with anti-MT antibody (1:100; clone E9, Zymed, Invitrogen) overnight. The next day, membranes were incubated with 1:10.000 horseradish peroxidase-HRP-conjugated secondary antibody (Santa Cruz, California, USA) in block buffer for 1 h (room temperature). Bound antibodies were visualized using BM Chemiluminesence Blotting Substrate POD (Roche, Brussels, Belgium) enhanced chemiluminesence detection kit, according to manufacturer's instructions. The membranes were exposed to X-ray films.

Boyden chamber chemotaxis assay

Supernatants from necrotic cells containing detectable MT on western blot were subjected to 0.5×10^6 leukocytes (isolated from a healthy volunteer using Ficoll-PaqueTM Plus [GE Healthcare Bioscience AB, Uppsala, Sweden]) in a Boyden trans-well assay with and without 100 µg/ml UC1MT anti-MT antibody (ThinCertTM 3µM pore size, TC Insert system, Greiner bio-one, Germany) [6]. Leukocytes migration through the membrane was counted in a Coulter chamber after 6 hours.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism® software (GraphPad Software, Inc., California, USA) or SPSS, version 21 for Windows (SPSS Inc., Illinois, USA). Data are presented as mean ± SEM. Differences between groups were calculated using an unpaired t-test or F test to compare variances in cases of normality or the Mann-Whitney test if the data were not normally distributed. Continuous data (body weight changes) were evaluated using linear mixed models. Kaplan-Meier survival curves were analysed using the log-rank

test (censored data). Correlations were calculated with the Spearman's rho test. A value of $p < 0.05$ was considered statistically significant.

Results

Colonic MT immunoreactivity correlates with the severity of human colitis

Colonic biopsies of IBD and acute infectious colitis patients were categorized based on histologic inflammation score and stained for MT. In biopsies of healthy subjects, MT was moderately to highly expressed in the epithelium, whereas a few immunopositive cells were observed in the lamina propria (Figure 1A). In contrast, biopsies from active colonic CD, UC and acute infectious colitis patients were characterized by an infiltration of MT positive inflammatory cells which correlated with the severity of colitis ($r = 0.643$ for colonic CD, $r = 0.328$ for UC and $r = 0.823$ for infectious colitis, Figure 1A and 1B). Epithelial MT expression was absent or decreased in highly necrotic regions in biopsies of acute infectious colitis and IBD patients, and there was no correlation between epithelial MT expression and the level of inflammation.

Metallothionein knockout mice are favoured in acute and chronic colitis

To investigate the role of MT in colitis, we compared the course of DSS-induced colitis in WT and MT-KO mice. Strikingly, only 10% of MT-KO mice died during acute colitis in contrast to 38% of the WT mice (cumulative percentage, Figure 2A). The higher survival rate in MT-KO mice was associated with less severe colonic inflammation, as demonstrated by a significant reduction of body weight loss, histological inflammation and colon shortening (Figure 2B-D). There was also less neutrophil infiltration in MT-KO compared to WT mice (based on reduced MPO activity levels without differences in infiltrating F4/80 positive macrophages) (Figure 2E and 2F).

Additionally, we investigated whether MT-KO mice were also favoured during chronic DSS-induced colitis. In accordance with results of the acute DSS model, we observed consistently higher body weights over time for MT-KO mice compared to WT mice (Figure 3A). No differences were found in survival rates (2 mice died in each group) or histological

inflammation, but the preserved colon lengths in MT-KO mice suggest a better defence against structural damage and remodelling in these animals (Figure 3B).

Anti-MT antibody treatment reduces signs of acute colitis

To transfer our findings to a clinically relevant model, we tested the therapeutic potential of the UC1MT anti-MT antibody in two acute colitis models. Reflecting the results of MT-KO mice, anti-MT treated mice tended to exhibit a higher survival rate and less body weight loss in DSS-colitis compared to placebo-treated mice (Figure 4A). Histological analyses of H&E-stained colon sections showed significantly less cell infiltration in UC1MT-treated mice compared to IgG-treated mice (Figure 4B). Differences were attributable to reduced macrophage infiltration, as demonstrated by lower F4/80 positive infiltrating cells in MT-KO mice (Figure 4C) without differences in MPO activity compared to control mice.

Finally, we evaluated the UC1MT treatment in TNBS-induced colitis. The UC1MT-treated mice showed again a trend of reduced weight loss compared to IgG-treated mice (Figure 5A). The UC1MT-treated mice again showed a significantly lower influx of F4/80 positive macrophages during active colitis compared to placebo-treated mice (Figure 5B and 5C). These results indicate that anti-MT antibody treatment reduces leukocyte infiltration in experimental colitis.

Anti-MT antibodies target the inflamed colon during colitis

To ensure that the therapeutic antibody is present at the colon, we used non-invasive *in vivo* small animal imaging to follow antibody distribution during DSS-colitis in mice injected with radio-active anti-MT antibody. Significantly more radioactivity was observed in colons of mice during active colitis. Colonic activity decreased during recovery; however, it still remained above baseline levels compared with colonic activity of control mice (mice injected on day 0) (Figure 6A and 5B). Additionally, autoradiography of proximal, mid and distal colon sections correlated with these results and showed radioactivity over the entire colon length (Figure 6C). These data demonstrate colonic accumulation of the UC1MT antibody after systemic administration during active colitis and clearance of the antibody during healing.

Extracellular endogenous released MT attracts leukocytes

Finally, we tested whether MTs can be released from a human colorectal cell line following different types of cell death. TNF induces receptor-dependent apoptosis, confirmed by increased caspase-3 activity after 6 and 24 hours. At 24 hours of TNF exposure, there was also increased LDH activity in cell supernatants, suggesting secondary necrosis following apoptosis [26], and this condition was associated with detectable MT in cell supernatants. Staurosporine, a receptor-independent apoptosis trigger, produced increased caspase-3 after 6 and 24 hours without LDH release and did not result in MT release. Repeated freeze/thawing induced a significant increased LDH activity, which was associated with an abundance of MT in cell supernatants (Figure 7A-C). In conclusion, MT release coincided with LDH release, suggesting that MTs are released from HT-29 cells upon necrosis.

Others have shown that supernatant from necrotic cells attracts leukocytes [27]. To investigate whether MT contributes to this leukocyte attraction, supernatants from necrotic cells were used in a chemotaxis assay, with and without anti-MT antibody. Significant leukocyte migration toward necrotic cell supernatant was observed, and the addition of UC1MT abolished this chemo-attraction. These results support the idea that endogenous released MT can act as a potent chemokine (Figure 7D).

Discussion

This study identifies a positive relationship between MT immunoreactivity and inflammation in human colitis. Moreover, MT inhibition improved experimental murine colitis. MT knockout mice were favoured in terms of survival and inflammation in both acute and chronic colitis. At the applied dose, anti-MT antibodies accumulate at the colon during colitis and are able to reduce macrophage infiltration with a trend towards an enhanced clinical outcome.

Our results show predominant MT immunoreactivity in epithelial cells and in the infiltrating cells during active colitis, whereas epithelial MT is absent in highly necrotic regions. Given the similar results observed in samples from IBD and acute infectious colitis patients, inflammation-mediated increase of MT positive inflammatory cells is not IBD-specific. The study of Bruwer *et al* aligns with our finding of increased MT immunoreactive

infiltrate during colitis, although they described a positive correlation between epithelial MT expression and inflammation [28]. Two other reports using immunohistochemistry on human colonic tissue describe decreased epithelial MT immunoreactivity in IBD patients compared to controls without MT detection in the lamina propria in IBD patients or controls [29,30]. The differences between the staining protocols described in the reported studies and ours relate to antigen retrieval [28,29] and primary antibody [30], factors that may influence MT immunoreactivity. Medication intake does not influence MT immunoreactivity, as previously reported [28]. Except for Ioachim and co-workers, who additionally included biopsies, the reported studies included surgical specimens whereas we exclusively used colonic biopsies. We can only speculate that tissue heterogeneity may be a factor contributing to the reported differences. Taken together, it can be concluded that MTs are predominantly expressed in the colonic epithelium and in inflammatory cells during active colitis.

The beneficial effect of genetic *Mt1* and *Mt2* deletion on survival and disease progression in our colitis model has previously been reported in an Alzheimer's disease model, in the TNF-induced lethal shock model and in hyperoxic acute lung injury [31,32,33]. Though, pro-survival properties for MTs have also been described. MT-KO mice were more sensitive to LPS/GalN-induced lethality and cardiac overexpression of MTs prolonged the mice life span [34,35]. It is not surprising that a protein with as many housekeeping roles to play will have different effects in different circumstances. Unfortunately, there are also conflicting results reported by others using MT-KO mice in DSS-induced colitis. Tran and co-workers saw less MPO activity and a tendency towards a lower disease activity in MT-KO mice compared to WT mice, which aligns our results [9]. In contrast, Oz and colleagues did not report a protective effect in MT-KO mice. Notably, in this study, the MT-KO was on a 129/Sv genetic background whereas MT overexpressing mice and wild type mice were from the C57BL/6J background [10]. Differences in susceptibility to experimental colitis among mouse strains has been reported. Comparisons are most readily interpreted if groups share the same genetic background [36]. Moreover, in the Oz study, mice were sacrificed after 1 week of DSS whereas we discovered a significant advantage at a later stage of disease (day 10). Recently, Tsuiji and colleagues reported an exacerbation of acute DSS-colitis in MT-KO mice [11]. These opposing results may be due to various factors. We used MT-KO mice

derived from a genetic construct originally produced by Palmiter's group [14] and used congenic WT mice sharing the same background as control. Tsuiji and co-workers used MT-KO mice produced in Choo's lab [37] and used WT mice originating from a different supplier. Although results reported by us and Tsuiji *et al* are both derived from C57BL/6 mice, reports in literature emphasize the impact of mice substrain and vendor on experimental outcome [36,38-40]. Besides the differences in genetic background between mice from different suppliers, microbial composition is also influenced by the environment and thus by the origin of the mice. Both factors, genetic background and gut microbiota, highly determine DSS-susceptibility and therefore, experimental groups should be sufficiently backcrossed and co-housed in order to minimize genomic and microbial heterogeneity among groups [36,38-44].

Consistently higher body weights in MT-KO mice during chronic DSS-induced colitis, confirmed the clinical benefit observed during acute colitis and resulted in a protection against structural damage and remodelling. We expanded our observation by using antibody-mediated MT inhibition in two models of acute colitis and found a significant reduction in the inflammatory infiltrate, predominantly affecting macrophages, with a tendency towards a beneficial effect on survival and weight loss. To localize the antibody during colitis, we subjected our mice to medical imaging after injection with radioactive UC1MT. The increased colonic radio-activity observed during active inflammation followed by a decrease at the time of mucosal healing, indicates a specific but reversible binding at the moment of epithelial damage and leukocyte infiltration and suggests the opportunity for its therapeutic application in human IBD.

Since therapeutic antibodies act on the extracellular pool of MT and excessive cell death is a hallmark of IBD, we investigated the release of MTs from apoptotic and necrotic HT29 cells. We could only detect MT in cell supernatant following necrosis, associated with plasma membrane damage and LDH release. Both forms of cell death, apoptosis and necrosis, have been implicated in IBD pathogenesis [2,44,45-51]. Apoptosis is referred to as a physiologically controlled process of cell death characterized by containment of cellular proteins within membranes and the silent removal of apoptotic bodies by phagocytosis which does not evoke inflammation. However, when the apoptotic rate exceeds phagocyte capacity, the unprocessed cells undergo secondary necrosis [3,27,52]. Necrosis, in contrast to apoptosis, results in plasma membrane rupture and the release of signals, recognized as

danger signals or alarmins, which are able to attract, activate and/or alert nearby immune cells of 'danger' to the host resulting in an inflammatory response [51,52]. Lynes *et al* previously described chemokine properties for exogenous MTs [6,7]. Our *in vitro* results support these observations. We identified endogenous released MT as a danger signal, attracting leukocytes towards necrotic cells and potentially damaging bystander cells to release more MT. Targeting extracellular MTs in IBD could dampen this vicious cycle. Indeed, as also shown for other danger signals such as high mobility group box 1 (HMGB1), blocking danger signals can result in partial suppression of the immune response and disease amelioration in murine IBD models [53]. By using MT knockout mice, we found that MTs are, as extracellular danger signals, not essential for initiation of the inflammation but that absence of MT dramatically reduced the severity of colitis.

Although MTs have well-known anti-oxidant properties [54], we here report the rather unexpected finding that inhibition of MTs is a strategy to dampen gut inflammation in murine colitis. However, since MTs are released upon intestinal epithelial cell damage and act as potent chemokines, this observation may not be too surprising [7,55-57]. Apparently, during colitis, the antioxidant properties of MTs are overruled by their detrimental role as danger signals. Therefore, we believe that inhibition of MT's bio-availability may be therapeutically valuable in immune-regulated pathologies that are associated with increased cell death.

In conclusion, preventive and therapeutic inhibition of the chemotactic function of MTs as danger signals comprises an effective strategy to reduce leukocyte infiltration and inflammation in murine colitis.

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Author contribution statement

LD, MDV, DL designed the study; LD conducted the experiments; LD, AW, CC, FDV, CV, DL contributed to acquisition of data; LD, PH, MAL, AW, CC, FDV, CV, MDV, DL: analysed data; LD: wrote the manuscript; PH, MDV, DL performed the critical revision of the manuscript.

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Table 1. Patients characteristics

		Control	Colonic CD	UC	Acute infectious colitis
Histological inflammation	No colitis	15	0	0	0
	Mild colitis	0	16	7	11
	Moderate colitis	0	10	5	5
	Severe colitis	0	11	8	0
Age in years (mean, range)		39 (24-63)	30 (11-57)	37 (13-68)	52 (15-82)
Gender	Female/male	5/10	25/12	10/10	6/10
Medication	No	15	18	3	15
	Immunosuppresives	0	2	1	0
	Corticosteroids	0	3	0	0
	Biologicals	0	1	0	0
	5-aminosalicylates	0	9	6	0
	Combination	0	4	8	0
	Missing	0	0	2	0

Figure legends

Figure 1. Active colitis is associated with metallothionein (MT) positive infiltrating cells in human colitis. (A) MT immunoreactivity in the colon of a healthy subject, an active colonic Crohn's disease (CD) and an active ulcerative colitis (UC) patient. Original magnification x 100. (B) Immunohistochemical scoring for lamina propria MT expression plotted against the degree of inflammation in colonic CD, UC and infectious colitis patients. Sections were scored for colitis and MT immunoreactivity by two independent observers and mean values are represented.

Figure 2. Suppression of DSS-induced acute colitis in metallothionein knockout (MT-KO) mice. (A) Kaplan-Meier survival and (B) weight curve of wild type (WT) and MT-KO mice treated with 4% DSS for 7 days, followed by 7 days of normal drinking water; *n* = 25. (C) Histological inflammation score, (D) colon length, (E) mucosal cell infiltration score and (F) myeloperoxidase activity of WT and MT-KO mice on day 10 of acute DSS-induced colitis; *n* = 5. Data are presented as the means ± SEM.

Figure 3. Enhanced outcome for metallothionein knockout (MT-KO) mice in chronic DSS-induced colitis. (A) Weight evolution and (B) colon length of wild type (WT) and MT-KO mice treated with 3 cycles of DSS/normal drinking water to induce chronic colitis; *n* = 14. Data are presented as the means ± SEM.

Figure 4. Anti-MT antibody (clone UC1MT) treatment in acute DSS-induced colitis. (A) Kaplan-Meier survival curve of IgG control and UC1MT-treated mice. (B) Histological scores of mucosal cell infiltrate and representative pictures of H&E-stained colon sections of IgG control and UC1MT-treated mice at day 10 of DSS-colitis. (C) Histological scores for mucosal F4/80 macrophage infiltration and representative pictures of F4/80-stained colon sections of IgG control and UC1MT-treated mice at day 10 of DSS-colitis. Data are presented as mean ± SEM; *n* = 9-11. Original magnification x 200.

Figure 5. Anti-MT antibody (clone UC1MT) treatment in acute TNBS-induced colitis. Mice treated with ethanol (EtOH) intrarectally served as controls for the TNBS/EtOH treatment.

(A) Weight evolution of IgG control and UC1MT-treated mice during TNBS-colitis. (B) Histological scores of mucosal cell infiltrate and representative pictures of H&E-stained colon sections of IgG control and UC1MT-treated mice at day 3 of TNBS-colitis. (C) Histological scores for mucosal F4/80 macrophage infiltration and representative pictures of F4/80 stained colon sections of IgG control and UC1MT treated mice during TNBS-colitis. Data are presented as mean ± SEM; *n* = 4-8. Original magnification x 200.

Figure 6. Anti-MT antibodies bind the colon during acute DSS-colitis. Antibody distribution was evaluated by injecting mice with radioactive UC1MT antibodies on days 0 (control, no colitis), day 7 (acute colitis), and day 14 (recovery) of DSS-induced colitis and subjecting them to a whole-body µSPECT/CT scan two days later. (A) Representative images of reconstructive SPECT/CT scans at the indicated time points. High activity in the kidneys and liver (interrupted arrows) represents blood clearance of the antibody. Full arrows indicate colonic binding. (B) Statistical analysis of the percentage injected dose per gram tissue (%ID/g) in the colon. The counts measured in the colon were normalized against the entire mouse body counts. Values are means ± SEM; *n* = 4-6 (C) Illustrative pictures of autoradiographic analyses of proximal, mid, and distal colon section at the indicated time points.

Figure 7. Metallothioneins (MTs) are released from necrotic HT29 cells and act as potent chemokines upon release. Fold increases as compared to untreated controls of (A) caspase-3/7 activity (luminescence) and (B) LDH activity of HT29 cell supernatant after 6 and 24 hours of treatment with TNF, staurosporine (Stauro), IFN (as control) and three freeze/thaw cycles. All conditions were preceded by 3 hours of zinc acetate treatment to increase endogenous MT levels and amplify the signal. Mean fold induction ± SEM values are shown; *n* = 3; *p<0.05; **p<0.01; ***p<0.0001 compared with 6 and 24 h in untreated cells. (C) MT (6 kDa) Western blot analyses of HT29 cell supernatant after the respective treatments. (D) Leukocyte attraction toward the MT-containing supernatant of necrotic HT29 cells was tested in a Boyden chamber assay with and without the addition of anti-MT antibody (UC1MT). Values are mean ± SEM; *n* = 3.

Figure 1

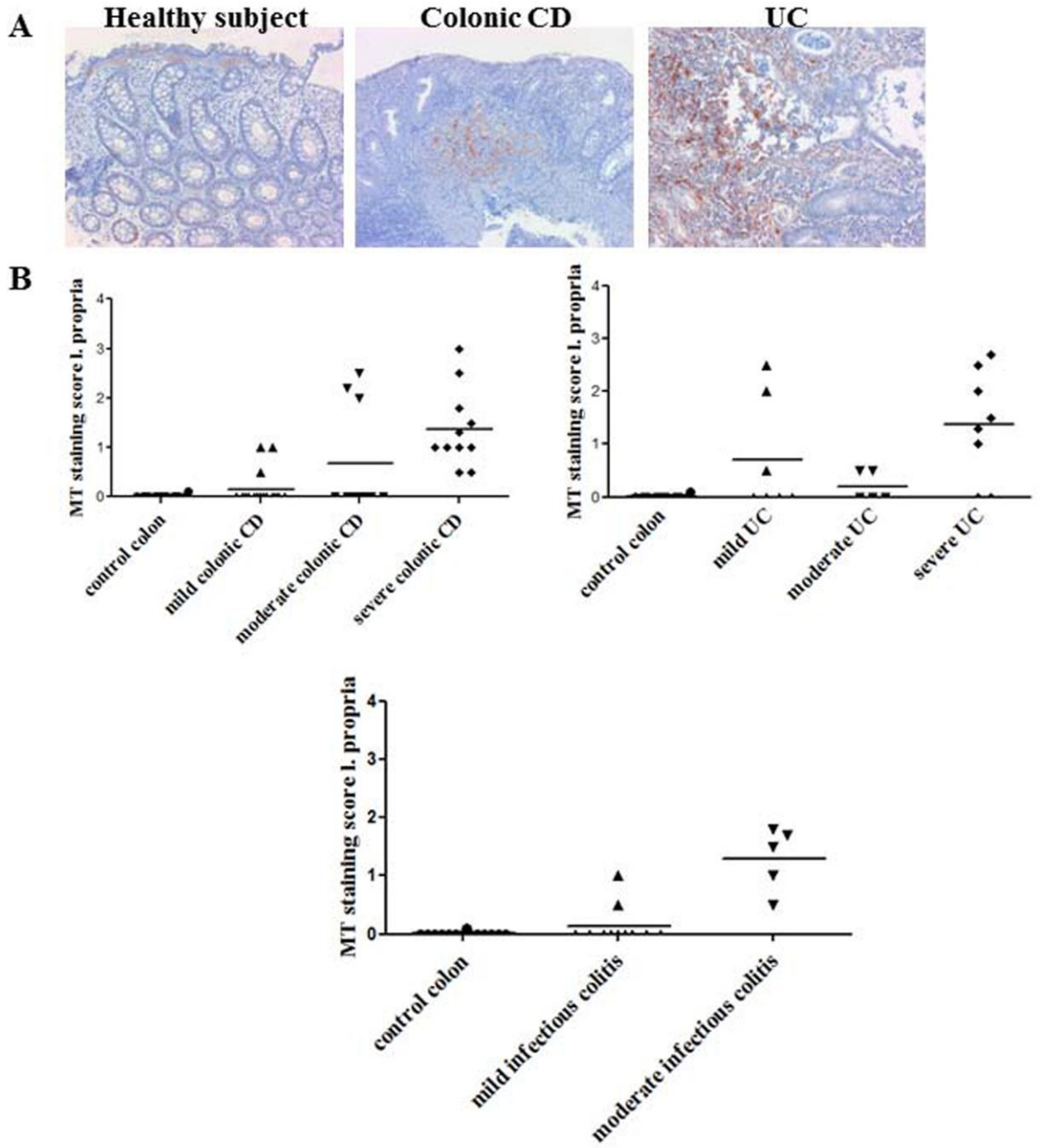

A
Healthy subject
Colonic CD
UC
B
MT staining score l. propria
control colon
mild colonic CD
moderate colonic CD
severe colonic CD
MT staining score l. propria
control colon
mild UC
moderate UC
severe UC
MT staining score l. propria
control colon
mild infectious colitis
moderate infectious colitis

Figure 2

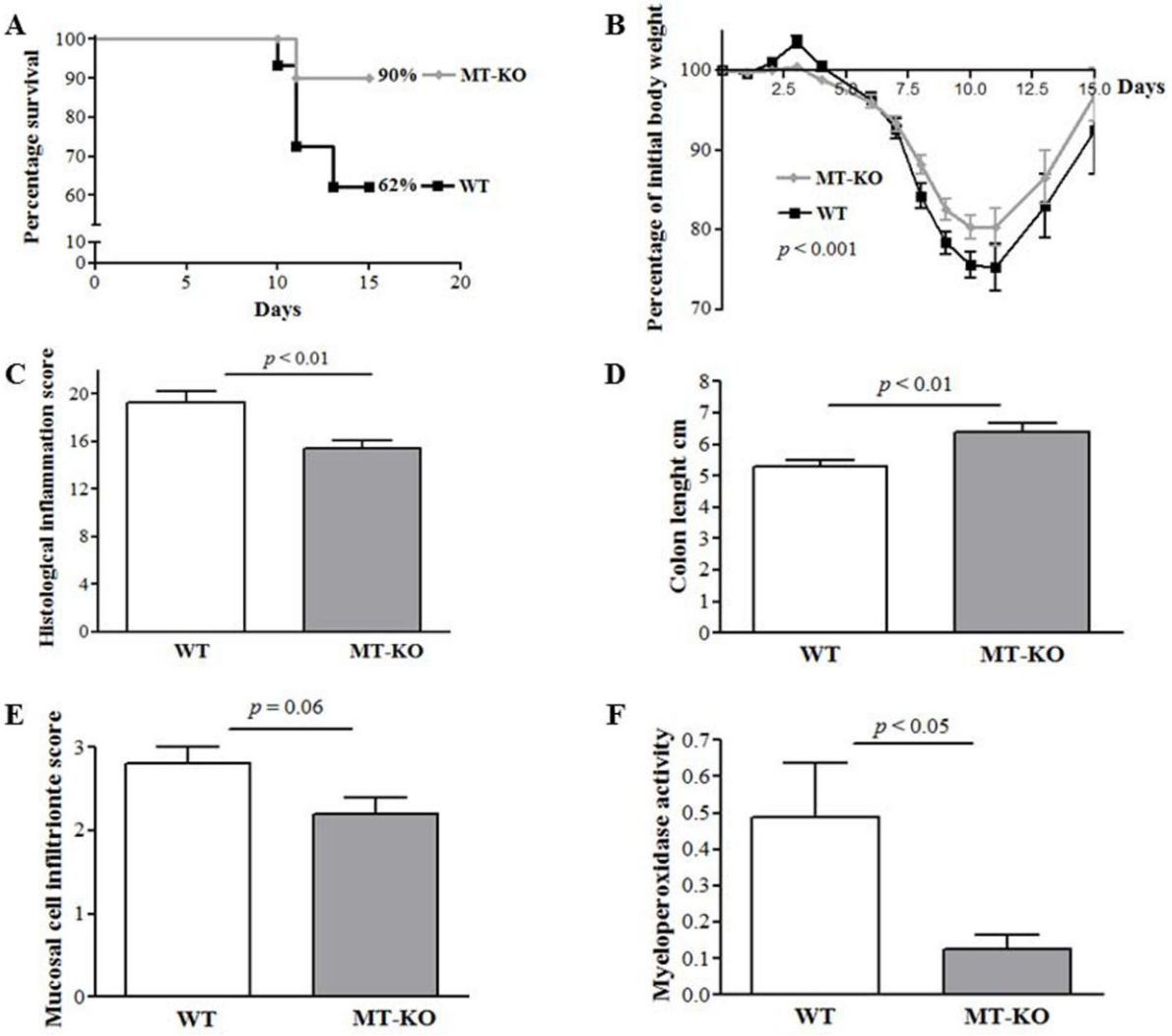

A
Percentage survival
100
90
80
70
60
10
0
0
5
10
15
20
Days
90%
MT-KO
62%
WT
B
Percentage of initial body weight
100
90
80
70
2.5
5.0
7.5
10.0
12.5
15.0
Days
MT-KO
WT
p < 0.001
C
Histological inflammation score
p < 0.01
20
16
12
8
4
0
WT
MT-KO
D
Colon lenght cm
p < 0.01
8
7
6
5
4
3
2
1
0
WT
MT-KO
E
Mucosal cell infiltrionte score
p = 0.06
3
2
1
0
WT
MT-KO
F
Myeloperoxidase activity
p < 0.05
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
WT
MT-KO

Figure 3

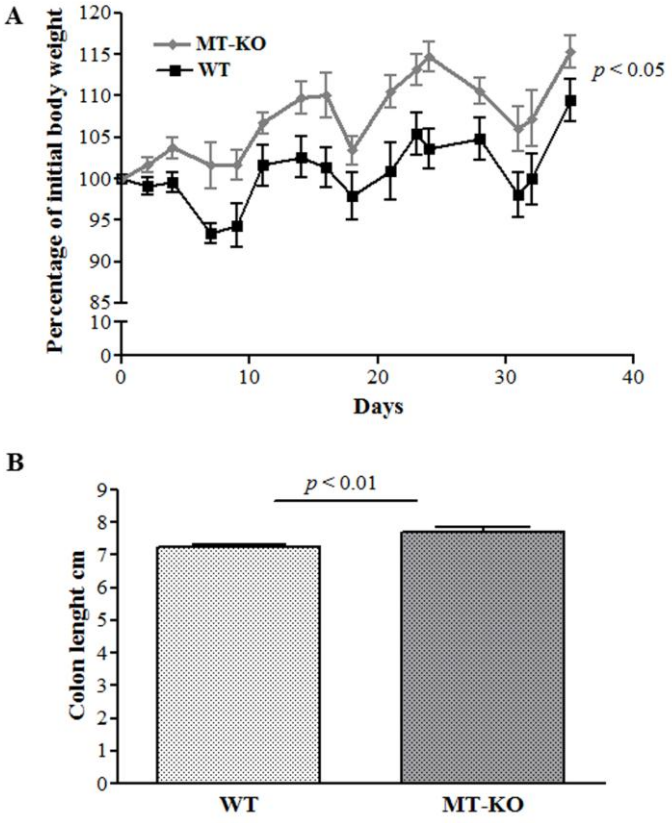

A
Percentage of initial body weight
MT-KO
WT
p < 0.05
Days
B
Colon lenght cm
p < 0.01
WT
MT-KO

Figure 4

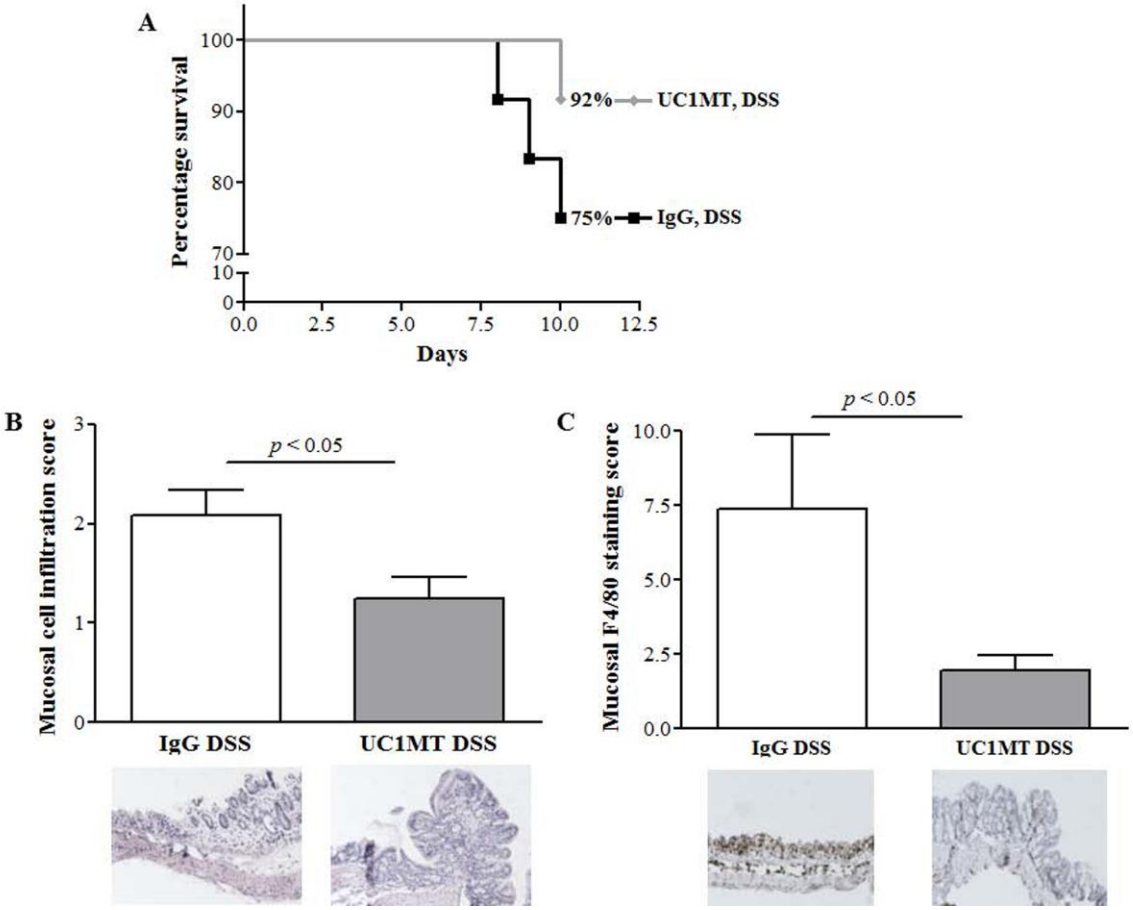

A
Percentage survival
100
90
80
70
10
0
0.0
2.5
5.0
7.5
10.0
12.5
Days
92%
UC1MT, DSS
75%
IgG, DSS
B
Mucosal cell infiltration score
3
2
1
0
p < 0.05
IgG DSS
UC1MT DSS
C
Mucosal F4/80 staining score
10.0
7.5
5.0
2.5
0.0
p < 0.05
IgG DSS
UC1MT DSS

Figure 5

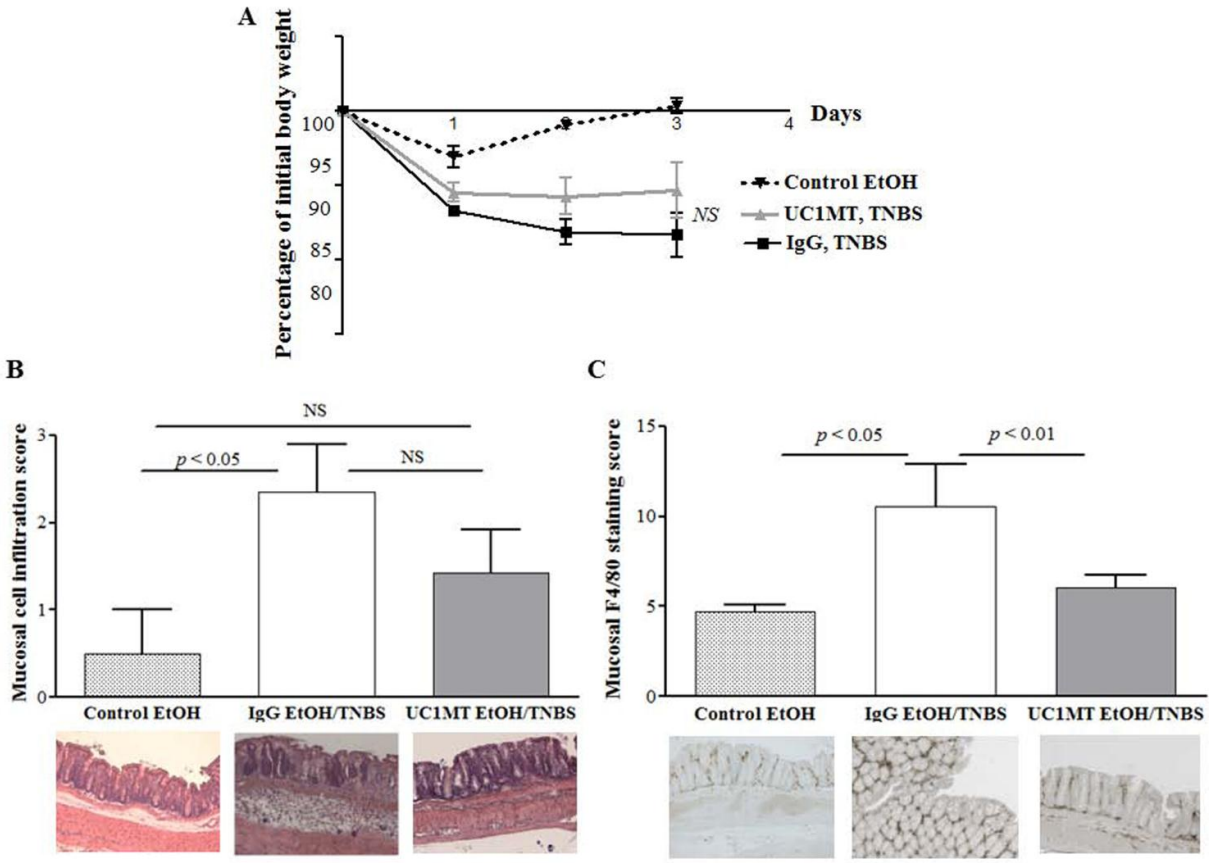

A
Percentage of initial body weight
100
95
90
85
80
1
3
4
Days
NS
Control EtOH
UC1MT, TNBS
IgG, TNBS
B
Mucosal cell infiltration score
3
2
1
0
NS
p < 0.05
NS
Control EtOH
IgG EtOH/TNBS
UC1MT EtOH/TNBS
C
Mucosal F4/80 staining score
15
10
5
0
p < 0.05
p < 0.01
Control EtOH
IgG EtOH/TNBS
UC1MT EtOH/TNBS

Figure 6

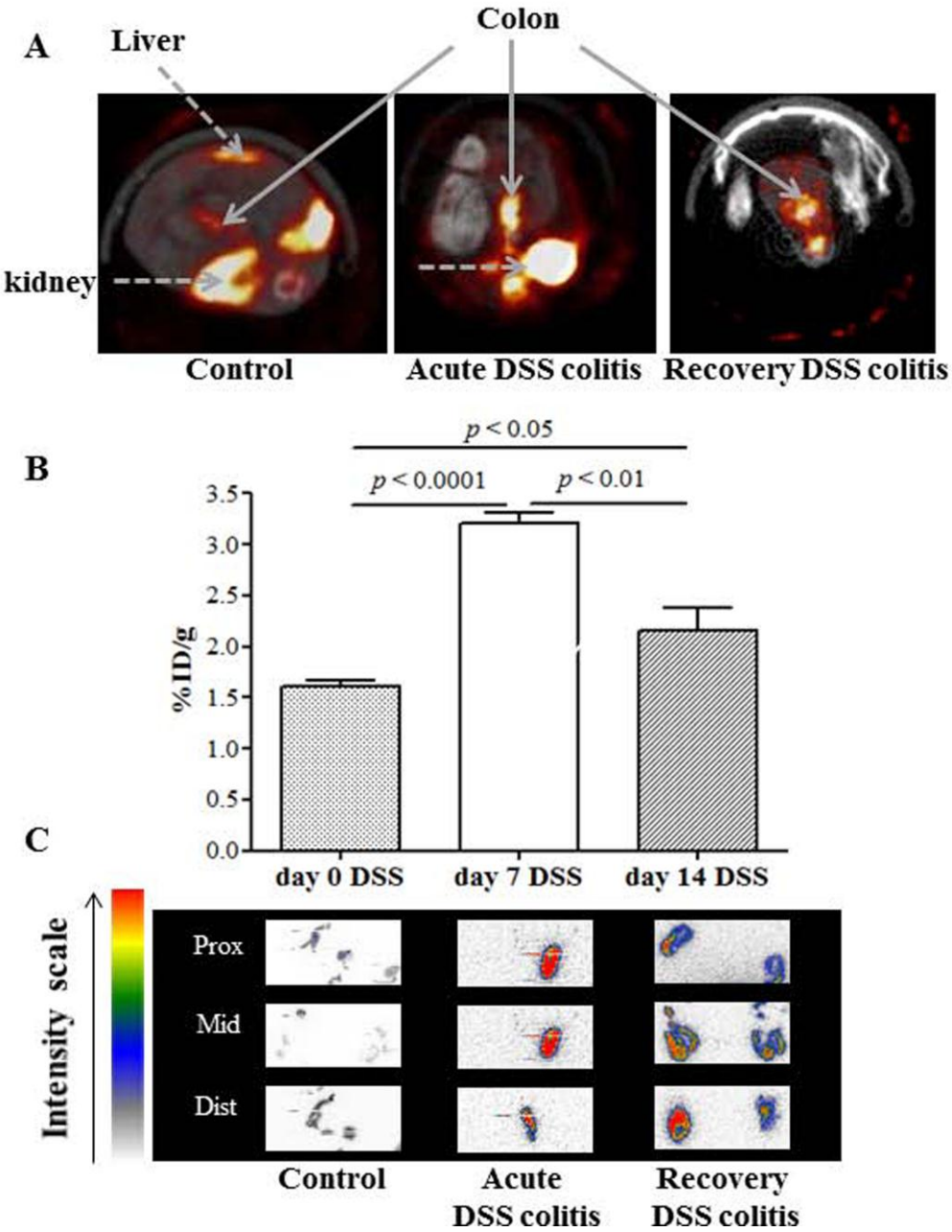
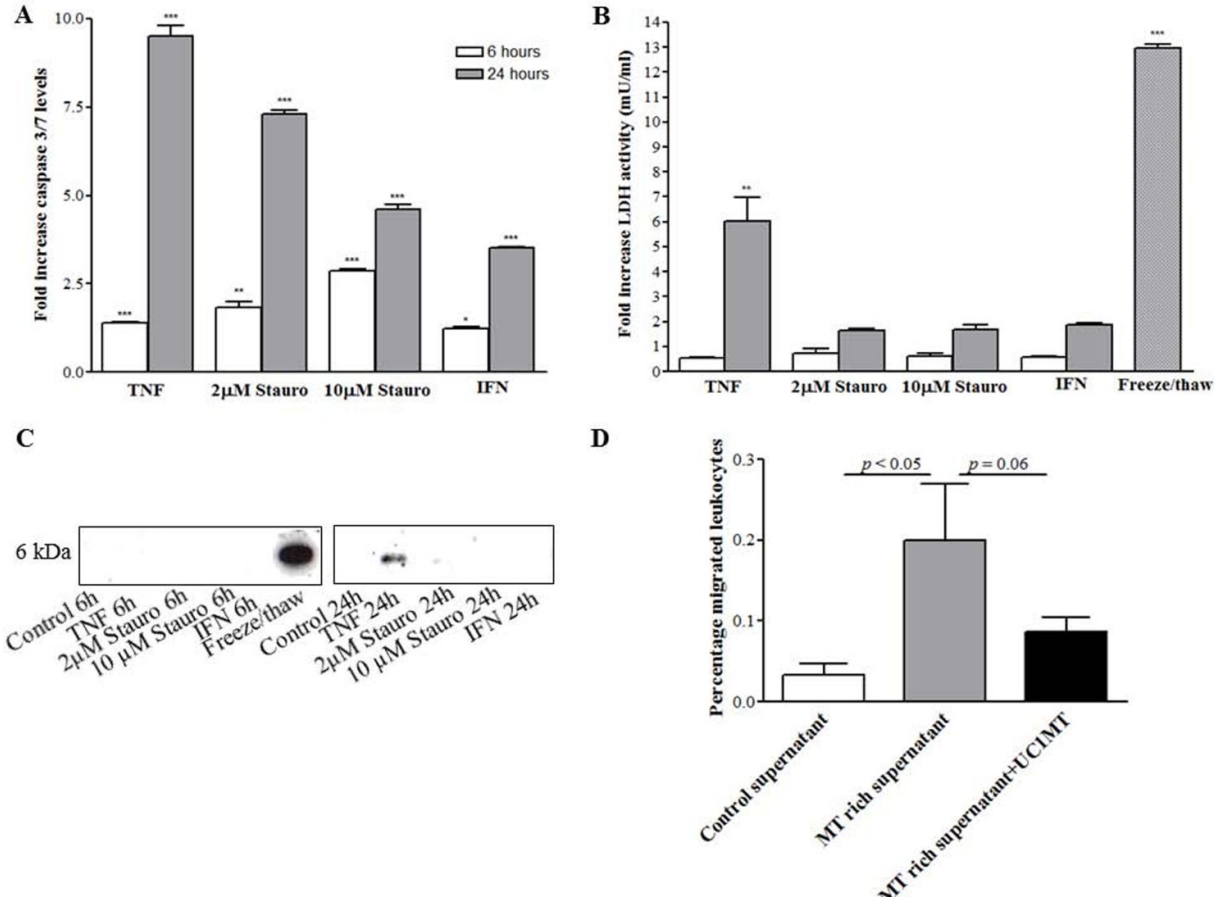

A
Liver
Colon
kidney
Control
Acute DSS colitis
Recovery DSS colitis
B
p < 0.05
p < 0.0001
p < 0.01
%ID/g
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
day 0 DSS
day 7 DSS
day 14 DSS
C
Intensity scale
Prox
Mid
Dist
Control
Acute DSS colitis
Recovery DSS colitis

Figure 7


A
Fold increase caspase 3/7 levels
6 hours
24 hours
TNF
2µM Stauro
10µM Stauro
IFN
B
Fold increase LDH activity (mU/ml)
TNF
2µM Stauro
10µM Stauro
IFN
Freeze/thaw
C
6 kDa
Control 6h
TNF 6h
2µM Stauro 6h
10 µM Stauro 6h
IFN 6h
Freeze/thaw
Control 24h
TNF 24h
2µM Stauro 24h
10 µM Stauro 24h
IFN 24h
D
Percentage migrated leukocytes
p < 0.05
p = 0.06
Control supernatant
MT rich supernatant
MT rich supernatant+UCIMT

Addendum to materials and methods

(1) Regarding the MT-KO and WT mice used in DSS-induced colitis:

Wild type (129S1/SvImJ) and MT-KO (129S7/SvEvBrd-Mt1tm1Bri Mt2tm1Bri/J) mice were originally purchased from The Jackson Laboratory by the group of Prof. Lynes (University of Connecticut) and backcrossed to C57BL/6J background for more than 10 generation. The wild type mice and MT-KO mice on the C57BL/6J background were provided by Prof. Lynes and further housed and reared at the laboratory animal facility at University Hospital Ghent. In order to avoid genetic drift, we routinely backcross MT-KO to wild type C57BL/6J, so we are confident that the wild type and MT-KO mice used in our experiments do not represent two substrains.

(2) Regarding the anti-MT antibodies used in the DSS- and TNBS-induced colitis and in *in vitro* experiments:

Clone UC1MT are monoclonal anti-MT antibodies, originally produced by the group of Prof. Lynes, which cross react with the isoforms MT1 and MT2. They are commercially available at Abcam (Cambridge, UK).

Chapter 3

Metallothioneins and Macrophage Polarization

Metallothioneins drive murine macrophages towards a pro-inflammatory phenotype

Manuscript in preparation

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Keywords: inflammation, interleukin-4, macrophage, metallothionein, Toll like receptor

Short title: Metallothioneins modulate macrophage polarization

ABSTRACT

Macrophages can roughly be divided into two functional groups, the classically activated M1 macrophages which respond to Toll like receptor (TLRs) ligands and the alternatively activated regulatory M2 macrophages which arise through interleukin-4 receptor alpha (IL-4Rα) signalling. During inflammation, pro-inflammatory M1 macrophages are responsible for the initial immune response, whereas M2 macrophages dampen excessive M1 activation and initiate tissue restoration. Metallothionein (MT) 1 and MT2 are immune-modulating proteins, which worsen the susceptibility to experimental colitis. Interestingly, intestinal inflammation is characterized by an infiltration of macrophages that express high amounts of MT1/2 and MT1/2 deletion decreases pro-inflammatory cytokine production following lipopolysaccharide (LPS) treatment in murine macrophages. The present study was set up to investigate whether the expression of MT1/2 influences M1/M2 signal transducers. Significantly lower mRNA expression of TLR4, Interferon regulatory factor 5 (IRF5) and IRF3 was found in untreated MT1/2 knockout (MT-KO) macrophages as compared to wild type (MT-WT) macrophages. Immunofluorescent staining confirmed low TLR4 levels in untreated and LPS-treated MT-KO macrophages and this phenotype was associated with an impaired M1 polarization following LPS. Conversely, only Krüppel-like factor 4 (KLF4), which facilitates M2 polarization, was significantly more expressed at mRNA level in MT-KO compared to MT-WT macrophages. Immunofluorescent staining additionally revealed higher levels of IL-4R in MT-KO macrophages, both basal and after IL-4 stimulation. Consequently, macrophages from MT-KO mice showed an enhanced IL-4 response compared to MT-WT macrophages. In conclusion, this study demonstrates that MTs influence M1/M2 signalling in murine macrophages by regulating the levels of M1/M2 signal transducers.

INTRODUCTION

Macrophages arise from the bone marrow as myelomonocytic precursor cells which differentiate to monocytes under interleukin-3 (IL-3), granulocyte macrophage-colony stimulating factor (GM-CSF) and M-CSF stimulation. Monocytes are released into the blood stream, migrate into tissue, differentiate and become tissue specific resident macrophages. Upon inflammation, macrophages are activated and new inflammatory cells are recruited. Inflammatory macrophages secrete pro-inflammatory cytokines and chemokines as well as reactive oxygen species, which is essential for proper defence and propagates the initial inflammatory reaction. However, these effector mechanisms may cause bystander damage and are therefore controlled by anti-inflammatory macrophages which promote wound healing and protect tissue integrity (Valledor et al. 1998; Murray & Wynn 2011). Both types of macrophages, the pro-inflammatory M1 type and the regulatory M2 type, comprise a large group of functional macrophages and their distinction is based on gene expression and cytokine/chemokine secretion after stimulation. The classically activated M1 macrophages respond to Toll-like receptor (TLR) ligands and interferon-gamma (IFN-γ) and secrete pro-inflammatory cytokines and chemokines such as IL-6, G-CSF and keratinocyte chemoattractant (KC) (Vellenga et al. 1988; Schaafsma et al. 1989; Larmonier et al. 2011; Murray & Wynn 2011; Tugal et al. 2013). Toll-like receptors are expressed by innate immune cells and function as sensors for pathogen-associated molecular patterns (known as PAMPs) and endogenous pathogenic signals (Janeway & Medzhitov 2002; Takeuchi & Akira 2010). Lipopolysaccharide (LPS) is a well-known stimulator of M1 macrophage polarization and LPS/TLR4 ligation results in MyD88-dependent and MyD88-independent pathway activation. The MyD88-dependent pathway activates the downstream transcription factor IFN regulatory factor 5 (IRF5) and nuclear factor-κB (NF-κB) which results in transcription of pro-inflammatory genes. The MyD88-independent pathway also activates NF-κB, however, together with IRF3 leading to the activation of Type I INFs and this further optimizes M1 polarization through the IFN feedback-loop (Figure 1) (Takaoka et al. 2005; Moynagh 2005; Honda & Taniguchi 2006; Lu et al. 2008; Tugal et al. 2013). The alternatively activated M2 macrophages are activated by IL-4 and IL-13, which both bind IL-4 receptor alpha (IL-4Rα) complex and signal through signal transducer and activator of transcription 6 (STAT6). After

phosphorylation and homodimerization of STAT6, IRF4 is recruited and induces the zinc-finger transcription factor Krüppel-like factor 4 (KLF4). The cooperation of STAT6 and KLF4 further promotes M2-associated gene transcription, such as arginase-1 (Arg-1) and chitinase-3 like 3 (Chi3l3 or Ym-1) (Figure 1) (Keegan et al. 1995; Kelly-Welch et al. 2003; El Chartouni et al. n.d.; Lawrence & Natoli 2011; Liao et al. 2011; Murray & Wynn 2011; Tugal et al. 2013).

The intestine covers the largest bulk of resident macrophages. Yet, during intestinal inflammation, monocytes are still recruited from the blood stream through a chemotactic gradient which is established by the production of chemokines from pro-inflammatory activated macrophages. We recently reported that these infiltrating inflammatory cells express high amounts of metallothionein (MT) 1 and MT2 , which are acute stress proteins, and that MT1/2 on itself are able to attract inflammatory cells (Yin et al. 2005; Devisscher et al. 2014). Metallothioneins are zinc binding proteins that were initially known for their protective function as heavy metal and radical scavenger. However to date, focus has set on the potential of MT1/2 to modulate immune cell function and immune responses (Borghesi & Lynes 1996; Lynes et al. 2014). The activation of pattern recognition receptors (PRRs) up-regulates MT1/2, which is essential for bacterial clearance, whereas MT1/2 deletion reduces bactericidal activity and pro-inflammatory cytokine production following LPS stimulation in murine macrophages (Youn et al. 1995; Kanekiyo et al. 2002; Sugiura et al. 2004; Itoh et al. 2005; Lahiri & Abraham 2014). In order to explore how intrinsic MT1/2 levels influence the functional outcome of macrophages, we assessed the levels of key M1/M2 transducers and their corresponding downstream signallers in bone-marrow derived macrophages of wild type (MT-WT) mice and mice with a targeted disruption of MT1 and MT2 (MT-KO).

MATERIAL AND METHODS

Mice

Wild type (129S1/SvImJ) and MT-KO (129S7/SvEvBrd-Mt1tm1Bri Mt2tm1Bri/J) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and housed and reared in the laboratory animal facility of Ghent University Hospital according to the institutional animal healthcare guidelines. Wild type and MT-KO mice were mated to produce heterozygotes which are mated inter se to produce wild type and MT-KO mice of the same

background. This study was approved by the Institutional Review Board of the Faculty of Medicine and Health Science of Ghent University (ECD 14/55).

Cells

Femora and tibia bone-marrow canals of MT-WT and MT-KO mice were flushed with cold sterile phosphate buffer saline to obtain bone-marrow derived macrophages. Cells were cultured in 10x15 mm petri-dishes in DMEM+Glutamax (Invitrogen, Merelbeke, Belgium) supplemented with 10% foetal calf serum, penicillin/streptomycin (Invitrogen) and recombinant murine macrophage colony stimulating factor (M-CSF, PeproTech, London, UK). Medium was changed every other day and cells were seeded for experiments on day 7. Cells were stimulated with 100 ng/ml LPS (Ultrapure LPS from E. coli K, Invivogen, San Diego, California, USA), 20 ng/ml murine recombinant IL-4 (PeproTech) or were left untreated for 24 hours. Cells were lysed for RNA extraction and supernatant was collected for cytokine/chemokine analyses.

Quantitative real-time PCR

Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Benelux, Venlo, the Netherlands) with on-column DNase treatment. The concentration and purity of the total RNA were determined using a spectrophotometer (WPA Biowave II, Isogen Life Science, the Netherlands). One microgram of total RNA was converted to single strand complementary DNA by reverse transcription (Superscript, Invitrogen) with oligo(dT) priming. The cDNA was diluted 1/8 and was used in real-time quantification with SYBR Green (Roche, Vilvoorde, Belgium) and 250 mM of each primer. A two-step program was run on a LightCycler® 480 (Roche, Vilvoorde, Belgium). Cycling conditions were 95°C for 10 minutes and 45 cycles of 95°C for 10 seconds and 60°C for 1 minute. A melting curve analysis confirmed primer specificity. All reactions were performed in duplicate and normalised to glyceraldehyde phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex subunit A (SDHA). The PCR efficiency of each primer pair was calculated using a standard curve from reference cDNA. The amplification efficiency was determined using the formula 10-1/slope. The primer pairs used are listed in table 1.

Cytokine measurements

Cytokines were measured in cell supernatant using the Bio-Plex MAGPIX multiplex reader (Bio-Rad, Hercules, California, USA) with a multiplex bead-based immunoassay kit for mouse IL-6, G-CSF, KC and IL-4. Samples were processed using the Bio-Plex Pro^{TM} Reagent Kit and analyzed with Bio-Plex Manager^{TM} Software.

Immunofluorescent staining

MT-WT and MT-KO macrophages were seeded at 10^5 cells/cm^2 in culture slides (BD Bioscience, Erembodegem, Belgium) and were treated with LPS, IL-4 or left untreated for 24 hours. Cells were fixed in 4% formaldehyde, permeabilized with 0,1% Triton X-100 (Sigma, Diegem, Belgium) and blocked in 1% bovine serum albumine (MP Biomedicals, Illkirch, France) for 10 minutes. Primary antibodies were applied overnight at 4°C (1/100 mouse monoclonal anti-TLR4, Abcam and 1/400 rabbit polyclonal anti-IL-4,antibodies online, Aachen, Germany). Alexa Fluor® 555 goat anti-mouse or Alexa Fluor® 488 goat anti-rabbit 1/200 (Life technologies Europe BV, Ghent, Belgium) was used as secondary antibody. Fluorescent signals were visualized and photographed using a Nikon TE300 epifluorescence microscope equipped with a 40 x oil-immersion objective (Plan APO, NA 0.45; Nikon) and a Nikon DS-Ri1 camera (Nikon Belux, Zaventem, Belgium).

Statistical analysis

Statistical analyses were performed using GraphPad Prism® software (GraphPad Software, Inc., California, USA). Data are represented as the mean ± SEM. Differences between groups were compared using an unpaired t-test for normally distributed data and the Mann-Whitney U test for data that were not normally distributed. Results with p values < 0.05 were considered statistically significant.

RESULTS

Metallothionein deletion dampens pro-inflammatory signaling in murine macrophages

Since previous reports describe an impaired LPS response in MT-KO macrophages compared to wild type macrophages (Kanekiyo et al. 2002; Sugiura et al. 2004), we first addressed the

intrinsic expression of TLR4 and the respective MyD88-dependent and independent downstream transcription factors, IFR5 and IRF3 in bone-marrow derived macrophages from MT-WT and MT-KO mice. Untreated macrophages from MT-KO mice express significantly lower mRNA TLR4, IRF5 and IRF3 compared to MT- WT macrophages (Figure 2A). Immunofluorescent staining confirmed lower TLR4 levels in MT-KO macrophages and this observation sustained following LPS stimulation (Figure 2B).

We additionally investigated if the observed low TLR4, IRF5 and IRF3 expression of MT-KO macrophages results in impaired M1 polarization. The secretion of the pro-inflammatory IL-6, G-CSF and KC was significantly induced following LPS treatment and this induction was significantly less pronounced in macrophages from MT-KO mice (Figure 3A-3B).

Metallothionein deletion facilitates M2 macrophage polarization

Next we investigated the effect of MT deletion on M2 macrophages phenotype. The basal expression of IL-4Rα and the downstream transcription factors IRF4 and KLF4 were analysed in MT-WT and MT-KO macrophages cultured overnight. KLF4 mRNA was significantly higher expressed in MT-KO macrophages compared to wild type macrophages (Figure 4A), but not IL-4R and IRF4. However, immunofluorescent staining showed higher IL-4R levels in MT knockout compared to MT-WT macrophages, both in untreated and IL-4 treated macrophages (Figure 4B).

We additionally assessed M2 polarization in MT-WT and MT-KO macrophages. IL-4 stimulation resulted in the induction of Chi3l3 expression and this induction was significantly more pronounced in MT-KO macrophages compared to wild type macrophages. Notably, IL-4 treatment evidently resulted in an increase of IL-4 cytokine in supernatant of both MT-WT and MT-KO macrophages, however, IL-4 concentrations were significantly more increased in supernatant of MT-KO macrophages which suggests an enhanced secretion compared to wild type macrophages (Figure 5).

DISCUSSION

The present study denotes the pro-inflammatory effect of MT1/2 on macrophage phenotype and their activation potential. MT1/2 deletion is associated with reduced TLR4 levels in

murine macrophages, resulting in an abrogation of downstream pro-inflammatory signalling. Vice versa, MT knockout macrophages display an M2 macrophage phenotype and an enhanced M2 response upon stimulation.

Previous research in murine derived MT knockout macrophages investigated the effect of MT1/2 on macrophage pro-inflammatory-associated functionality. Kanekiyo and colleagues report a reduction of NF-κB activity and TNF secretion following LPS treatment in MT-KO macrophages and additionally reported reduced bactericidal activity and nitric oxide production in MT knockout macrophages (Kanekiyo et al. 2002; Itoh et al. 2005). Sugiura and co-workers describe a defective phagocytic and antigen presenting activity and reduced pro-inflammatory cytokine production in MT-KO macrophages (Sugiura et al. 2004). MT down-regulation using an antisense MT mRNA vector resulted in the same reduction of LPS-triggered monocyte activation (Leibbrandt et al. 1994). Recent discoveries even reported MT1/2 up-regulation as a critical factor for enhanced bacterial clearance upon PRR overstimulation in macrophages (Lahiri & Abraham 2014). Our results align with these observations. We show an impaired induction of pro-inflammatory markers upon LPS stimulation and extend the knowledge on how intracellular MT1/2 exert their effect on macrophage signature. MT1/2 deletion results in reduced TLR4 and increased IL-4Rα levels. Both signal transducers dominate LPS and IL-4 signalling pathways, and alterations in these receptors may readily affect downstream transcription factors and effector signals. Indeed, IRF5 and IRF3 were expressed at significantly lower levels in MT-KO macrophages and MT-KO macrophages encompass a reduced sensitivity to MyD88-dependent and independent pathway activation (Figure 1). Indeed, IRF5 has been denoted as M1/M2 regulator since overexpression induces M1 and supresses M2 gene transcription (Krausgruber et al. 2011). Conversely, KLF4 was significantly higher expressed in MT-KO macrophages and overexpression of KLF4 facilitates M2 conversion (Liao et al. 2011). If MT1/2 directly influence transcriptional regulation of TLR4/IL-4R and affect downstream factors secondarily or if they exert their effect through transcription factors that in turn regulate receptor transcription remains to be investigated. It has been shown that NF-κB controls transcriptional activation and posttranscriptional stabilization of TLR4 and that MTs induce NF-κB activity (Abdel-Mageed & Agrawal 1998; Kanekiyo et al. 2001). The absence of tonic MT stimulation on NF-κB activity in MT knockout macrophages could therefore account for

the reduced TLR4 levels (Yan 2006). Furthermore, LPS induces MT1/2 expression which may further enhance NF-κB transcriptional activity, pro-inflammatory cytokine production and TLR4 expression (Leibbrandt & Koropatnick 1994).

Importantly, the present results add further knowledge on how MTs might be involved in propagating – intestinal – inflammation. We previously reported decreased susceptibility to colitis in mice with reduced MT1/2 levels (Devisscher et al. 2014). MT knockout mice showed an enhanced survival and reduced signs of colitis. TLR4 and MyD88 are essential for bacterial defence and control the oral tolerance (Araki et al. 2005; Fukata et al. 2005; Fukata et al. 2006). However, when epithelial integrity is damaged, microbial infiltration into the lamina propria results in an excessive TLR4 stimulation. The subsequent rise in pro-inflammatory cytokine secretion results in substantial bystander damage during on-going inflammation. Regulatory macrophages are therefore necessary to dampen the initial reaction, to promote recovery and to restore tissue integrity. Macrophages from MT knockout mice with reduced TLR4/IRF5/3 and enhanced IL-4Rα/KLF4 levels might more efficiently turn on their regulatory program and accelerate recovery.

Collectively, we show that intracellular MTs influence M1/M2 signalling in murine macrophages by regulating the levels of M1/M2 signal transducers.

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CONFLICT OF INTEREST

Lindsey Devisscher, Martine De Vos and Debby Laukens are listed as co-inventors on a patent application protecting the use of MT antagonists to treat intestinal inflammation (WO2013007678).

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FIGURE LEGENDS

Figure 1. Schematic overview of M1/M2 signalling in murine macrophages.

Arg-1, arginase-1; Chi3l3, chitinase-3 like 3; G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; IRF, INF regulatory factor; KC, keratinocyte chemo-attractant; KLF4, Krüppel-like factor 4; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor-κB; STAT, signal transducer and activator of transcription; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TRIF, Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β.

Figure 2. Metallothionein promotes the M1 phenotype in murine macrophages

(A) Basal mRNA expression of Toll-like receptor 4 (TLR4), Interferon regulatory factor (IRF) 5 and IRF3 in wild type (MT-WT) and MT knockout (MT-KO) bone-marrow derived macrophages cultured for 24 hours. Results are representatives of two independent experiments, each in triplicate. *P<0.05. (B) Representative images of TLR4 immunofluorescent stainings in MT-WT and MT-KO macrophages cultured for 24 hours in control medium and medium supplemented with 100 ng/ml lipopolysaccharide (LPS).

Figure 3. Metallothionein promotes M1 macrophage polarization

(A) Interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF) and keratinocyte chemoattractant (KC) concentrations in supernatant of wild type bone-marrow derived macrophages treated with 100 ng/ml lipopolysaccharide (LPS) or 20 ng/ml interleukin-4 (IL-4). (B) Fold inductions of IL-6, G-CSF and KC cytokine secretion in supernatant of wild type (MT-WT) and MT knockout (MT-KO) macrophages following LPS stimulation. Results are representatives of two independent experiments, each in triplicate. **P<0.01; ***P<0.001.

Figure 4. Metallothionein deletion promotes the M2 phenotype in murine macrophages

(A) Basal mRNA expression of interleukin-4 receptor alpha (IL-4Rα), Interferon regulatory factor 4 (IFR4) and Krüppel-like factor 4 (KLF4) in wild type (MT-WT) and MT knockout (MT-KO) bone-marrow derived macrophages cultured for 24 hours. Results are representatives of two independent experiments, each in triplicate. *P<0.05. (B) Representative images of IL-4R

immunofluorescent stainings in MT-WT and MT-KO macrophages cultured for 24 hours in control medium and medium supplemented with 20 ng/ml IL-4.

Figure 5. Metallothionein deletion promotes M2 macrophage polarization

(A) mRNA expression of chitinase-3 like 3 (Chi3l3) and interleukin-4 (IL-4) concentration in supernatant of wild type bone-marrow derived macrophages treated with 100 ng/ml lipopolysaccharide (LPS) or 20 ng/ml interleukin-4 (IL-4). (B) Fold inductions of mRNA Chi3l3 expression and IL-4 cytokine secretion in supernatant of wild type (MT-WT) and MT knockout (MT-KO) macrophages following IL-4 stimulation. Results are representatives of two independent experiments, each in triplicate. *P<0.05; **P<0.01; ***P<0.001.

Table 1. Sequences and qPCR efficiencies of the primer sets used for quantitative real time-PCR

Gene symbol	Forward primer	Reverse primer	Efficiency %	R^2
Mouse				
GAPDH	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA	85	0,99
SDHA	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT	103	0,99
TLR4	GCTTACACCACCTCTCAAACTT	AACTTCCTGGGGAAAAACTCTG	97	0,99
IL-4Rα	TGGAAGTGCGGATGTAGTCAG	TGGATCTGGGAGCATCAAGGT	97	0,99
IRF5	AGAGACAGGGAAGTACACTGAAG	TGGAAGTCACGGCTTTTGTTAAG	95	0,99
IRF3	GAGAGCCGAACGAGGTTCAG	CTTCCAGGTTGACACGTCCG	100	0,99
IRF4	TCCGACAGTGGTTGATCGAC	CCTCACGATTGTAGTCCTGCTT	107	0,99
KLF4	GTGCCCCGACTAACCGTTG	GTCGTTGAACTCCTCGGTCT	95	0,99
Chi3l3	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA	95	0,99

Figure 1

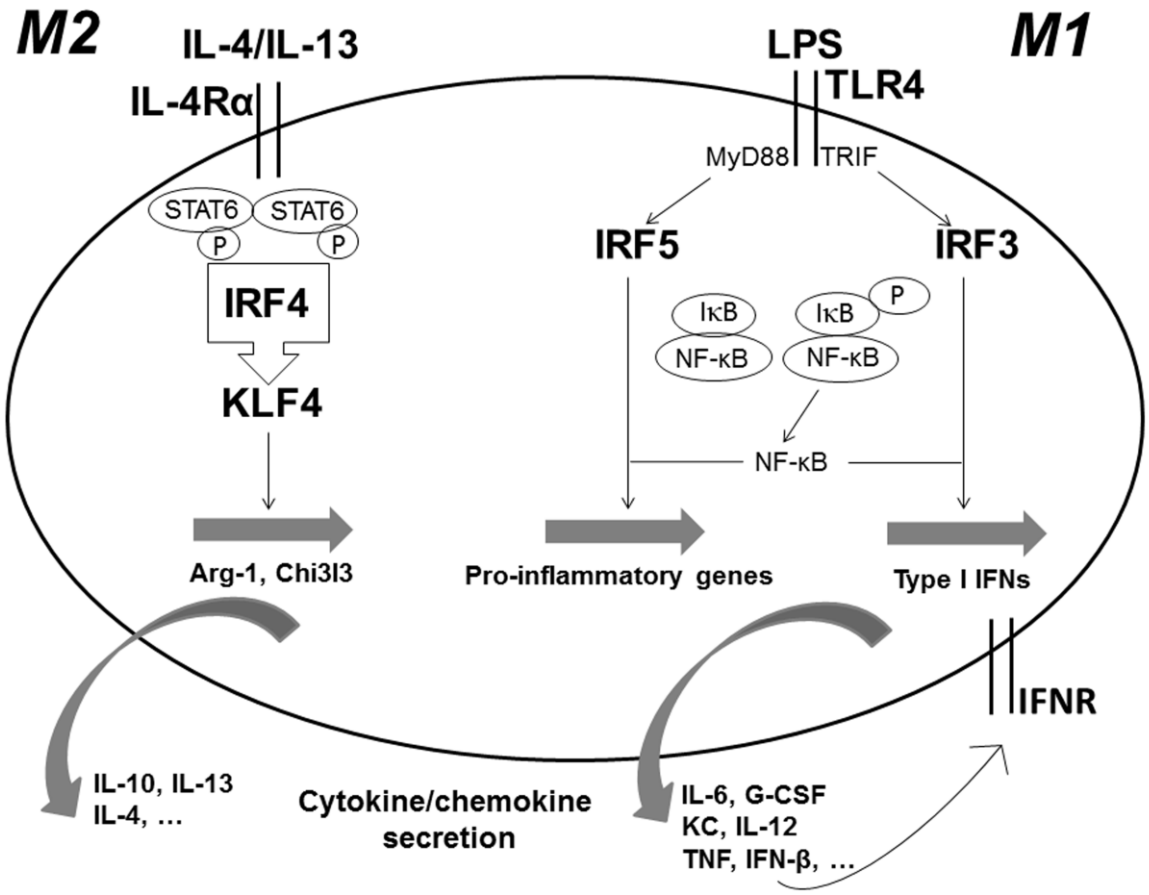
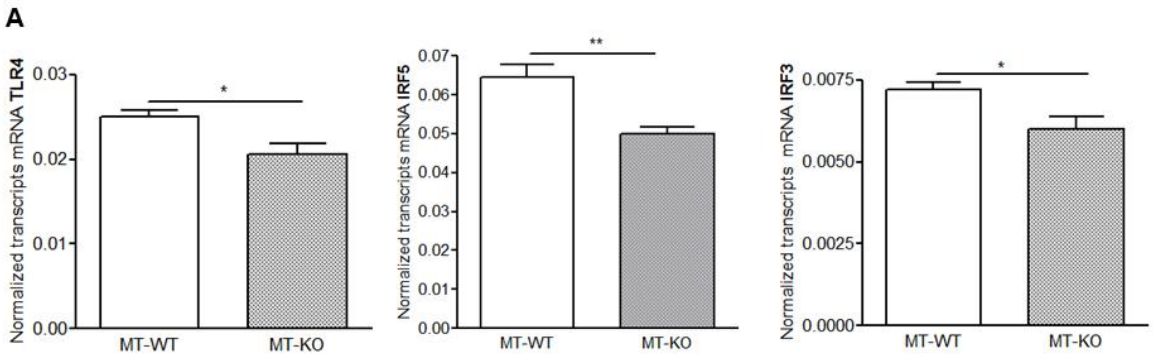

M2
IL-4/IL-13
IL-4Rα
STAT6
STAT6
P
P
IRF4
KLF4
Arg-1, Chi3l3
IL-10, IL-13
IL-4, …
M1
LPS
TLR4
MyD88
TRIF
IRF5
IRF3
IκB
NF-κB
IκB
P
NF-κB
NF-κB
Pro-inflammatory genes
Type I IFNs
IFNR
Cytokine/chemokine
secretion
IL-6, G-CSF
KC, IL-12
TNF, IFN-β, …

Figure 2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0.02
0.01
0.00
*
MT-WT
MT-KO
Normalized transcripts mRNA IRF5
0.07
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0.05
0.04
0.03
0.02
0.01
0.00
**
MT-WT
MT-KO
Normalized transcripts mRNA IRF3
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0.0050
0.0025
0.0000
*
MT-WT
MT-KO

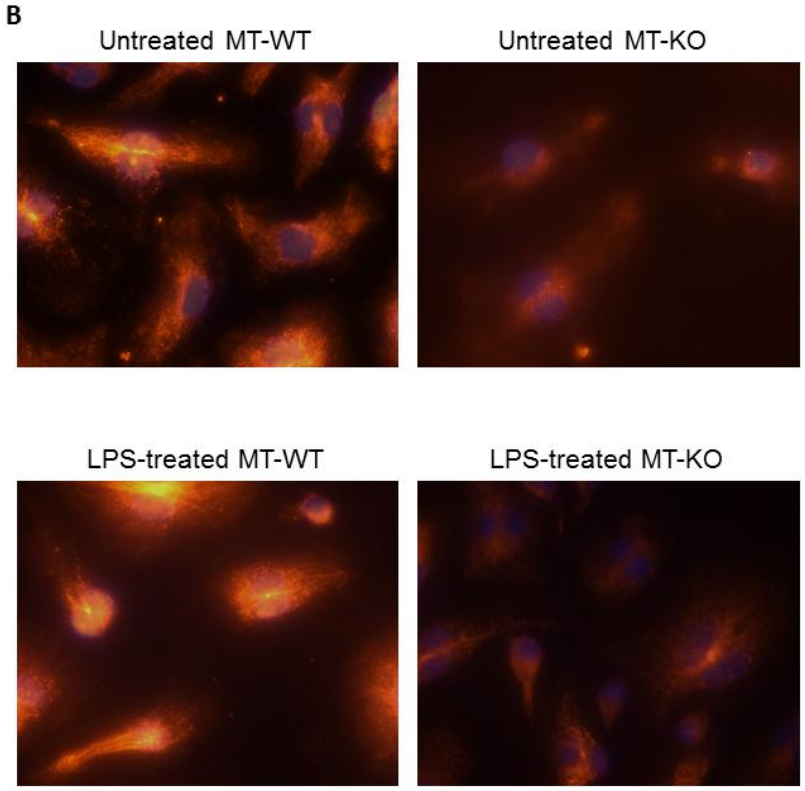
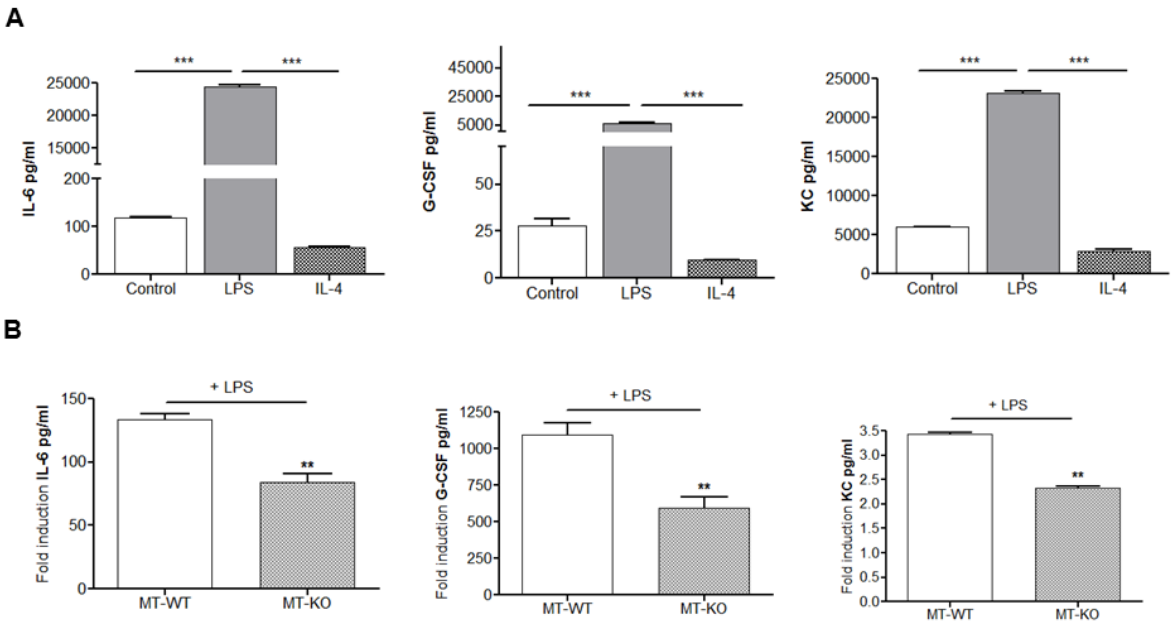

B
Untreated MT-WT
Untreated MT-KO
LPS-treated MT-WT
LPS-treated MT-KO

Figure 3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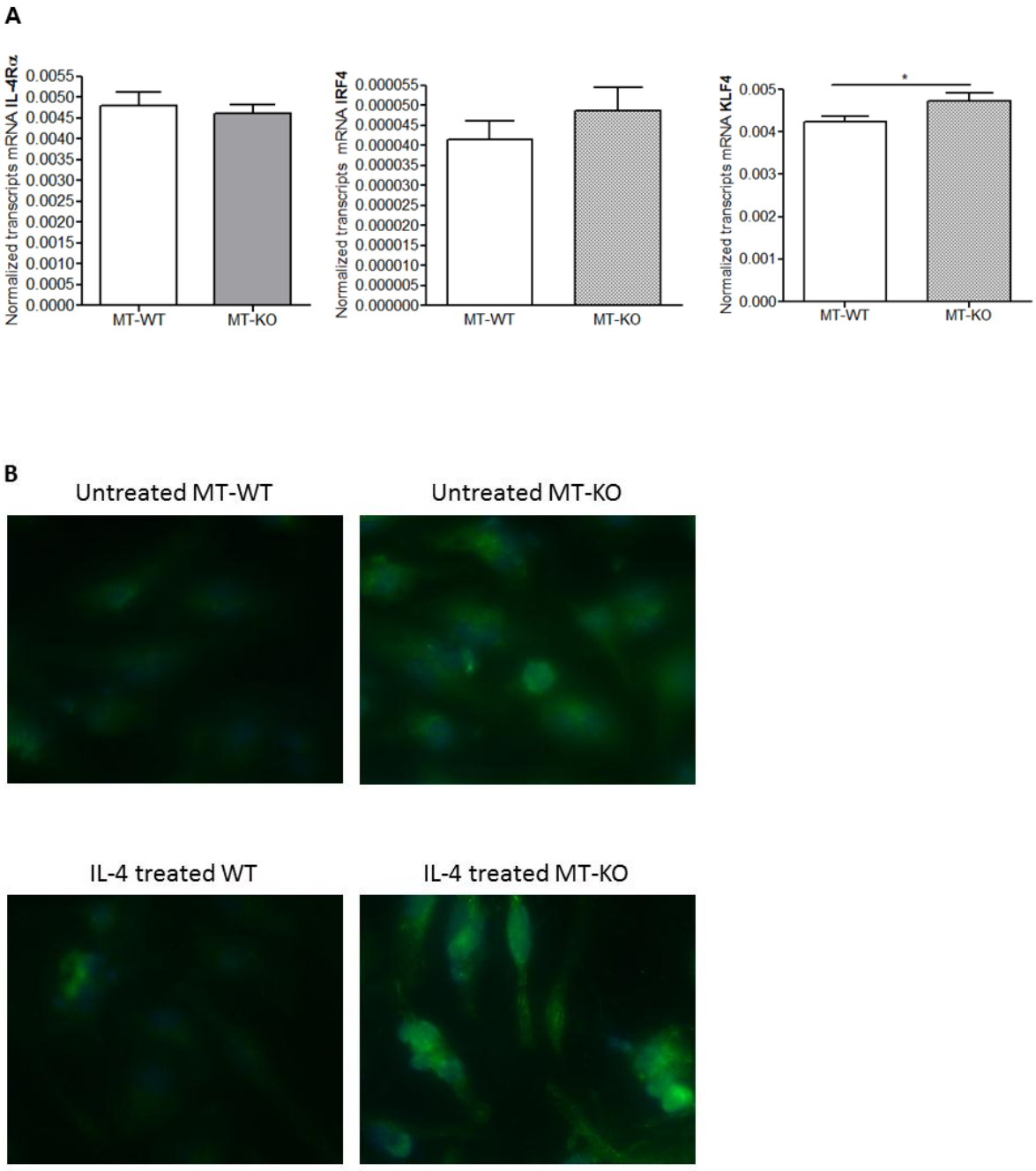
IL-6 pg/ml
25000
20000
15000
200
100
0
***
***
Control
LPS
IL-4
G-CSF pg/ml
45000
25000
5000
50
25
0
***
***
Control
LPS
IL-4
KC pg/ml
25000
20000
15000
10000
5000
0
***
***
Control
LPS
IL-4
B
Fold induction IL-6 pg/ml
150
100
50
0
+ LPS
**
MT-WT
MT-KO
Fold induction G-CSF pg/ml
1250
1000
750
500
250
0
+ LPS
**
MT-WT
MT-KO
Fold induction KC pg/ml
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
+ LPS
**
MT-WT
MT-KO

Figure 4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0.0050
0.0045
0.0040
0.0035
0.0030
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0.0020
0.0015
0.0010
0.0005
0.0000
MT-WT
MT-KO
Normalized transcripts mRNA IRF4
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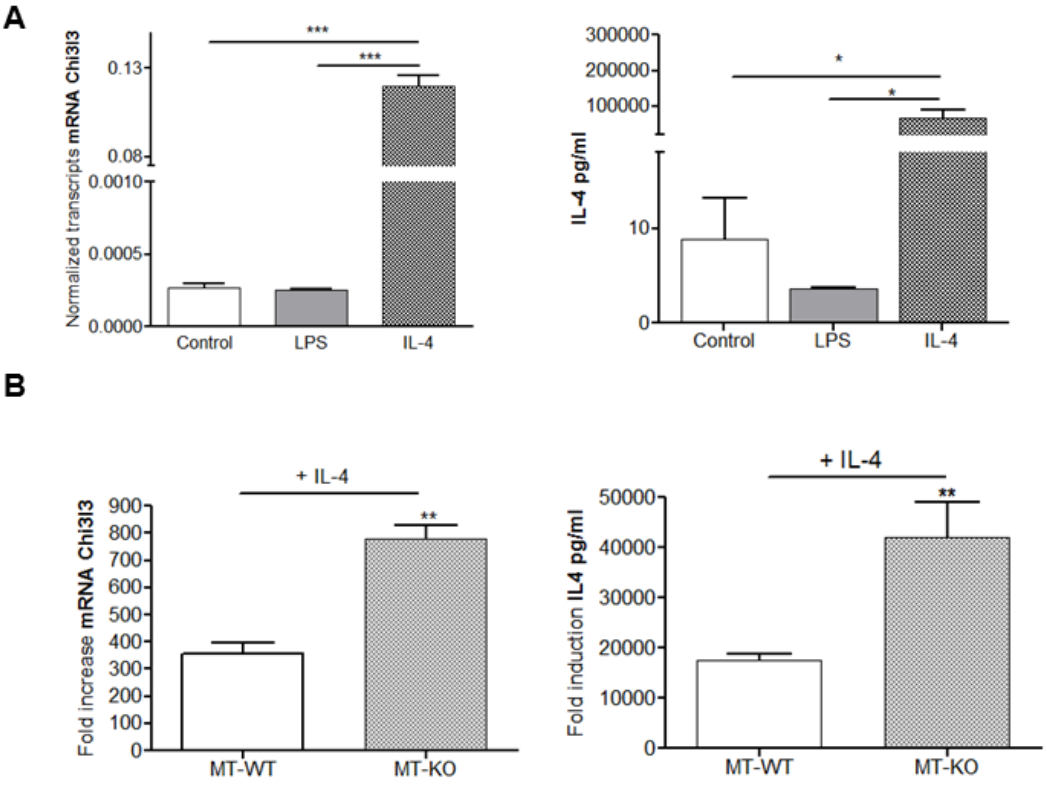
0.000050
0.000045
0.000040
0.000035
0.000030
0.000025
0.000020
0.000015
0.000010
0.000005
0.000000
MT-WT
MT-KO
Normalized transcripts mRNA KLF4
*
0.005
0.004
0.003
0.002
0.001
0.000
MT-WT
MT-KO
B
Untreated MT-WT
Untreated MT-KO
IL-4 treated WT
IL-4 treated MT-KO

Figure 5


A
Normalized transcripts mRNA Chi3l3
0.13
0.08
0.0010
0.0005
0.0000
***
***
Control
LPS
IL-4
IL-4 pg/ml
300000
200000
100000
10
0
*
*
Control
LPS
IL-4
B
Fold increase mRNA Chi3l3
900
800
700
600
500
400
300
200
100
0
+ IL-4
**
MT-WT
MT-KO
Fold induction IL4 pg/ml
50000
40000
30000
20000
10000
0
+ IL-4
**
MT-WT
MT-KO

Discussion and Future perspectives

Discussion

Metallothionein 1/2 are acute stress proteins with immune-modulating functions. They participate in the pathogenesis of different inflammatory conditions and a role for MT1/2 in intestinal inflammation had been proposed, however without conclusive results. This work aimed at elucidating the role of MT1/2 in experimental colitis and providing further evidence for these isoforms as immune-regulating proteins in intestinal inflammation. This discussion is based on actual data provided in chapter 1, 2 and 3. In the future perspectives we elaborate on the unresolved issues of the presented work and provide some preliminary data of future projects.

I. Metallothioneins and the hypoxic adaptive response

Inflammation-induced hypoxia is a characteristic feature of a number of inflammatory conditions and contributes to the perpetuation of the inflammatory reaction and delay in tissue recovery. This is partly mediated by the increased metabolism of infiltrating cells and vasculitis. In the intestine, a high oxygen gradient exists from the well-perfused and oxygenated sub-epithelial mucosae to the anoxic luminal part. Therefore, the intestinal epithelium encounters a lower oxygen tension compared to other tissue, rendering them in a state of 'physiological hypoxia', and it is intelligible that these cells are the most prone to a further decrease in oxygen supply [1]. Cells are equipped with a hypoxia-induced mechanism that enables them to respond and adapt to reduced oxygen supply. This pathway involves the stabilization of HIF-1α which induces the transactivation of pro-angiogenic genes, such as VEGF and EPO. Activation of this hypoxic response has proven to be beneficial in IBD mouse models [2,3]. Human IBD is characterized by increased HIF-1 levels and MT1/2 are known for their protective intracellular properties. Initially we speculated that the positive correlation between HIF and MT1/2, described in other inflammatory diseases, would also be present and advantageous in intestinal inflammation. The potential to manipulate this HIF/MT relation in order to abrogate the on-going inflammation, formed initially the basis of this thesis.

In **chapter 1**, we investigated the relation between MT1/2 and HIF-1α in intestinal epithelial cells. Surprisingly, we found that HIF-1α stabilization after DMOG treatment resulted in a down-regulation of mRNA MT expression in the HT-29 celline, in mouse as well as in human isolated colonocytes. By using SiRNA targeting HIF and MT1/2, we demonstrated that HIF down-regulates MT1/2 expression and that MT1/2 are in turn able to suppress HIF-1α stabilization. Intracellular zinc chelation inhibited the formation of functional HIF-1α and prevented MT1/2 down-regulation following DMOG, demonstrating the role of zinc in HIF-mediated MT1/2 down-regulation. Subsequently, SiRNA-MT treated cells have increased free zinc levels [4] which can be applied for HIF-1α stabilization in case of PHD inhibition. The latter might account for the increased HIF-1α stabilization observed in SiRNA-MT cells compared to cells with high MT1/2 levels. The inverse HIF/MT relation was also present during DSS-induced colitis. Metallothionein 1 increased during the peak of inflammation, which is consistent with their identification as acute phase protein. The observed decreased expression of VEGF might come from MT's mitigating impact on HIF induction but could also indicate that neo-angiogenic mechanisms are not yet activated at this stage of intestinal inflammation.

II. Metallothioneins and experimental colitis

Since HIF-1α stabilisation is beneficial in IBD mouse models [2,3], the HIF/MT inverse correlation questioned the proposed protective role of MT1/2 in intestinal inflammation. Indeed, in **chapter 2** we demonstrated the beneficial effects of reducing MT1/2 in the outcome of experimental colitis. Complete elimination of *Mt1* and *Mt2* resulted in reduced susceptibility to colitis in MT-KO mice, both in an acute and chronic setting. Partial neutralisation of released MT1/2 by the use of anti-MT antibodies targeting the isoforms MT1 and MT2, reduced signs of colitis in acute DSS and TNBS colitis. Importantly, systemically administrated radioactively labelled antibodies were detected at the inflamed colon on microSPECT/CT scans of mice and colonic radioactivity was less pronounced during mucosal healing. This strengthens the potential for therapeutic use of anti-MT1/2 antibodies in human colitis. Since anti-MT antibodies only act extracellular, we tested the release of

MT1/2 following apoptotic and necrotic triggers *in vitro*. Extracellular MT1/2 could only be detected after necrotic cell death and was partly responsible for the chemotactic effect of necrotic cell supernatant, demonstrated by anti-MT1/2 antibodies. The release of MT1/2 following necrosis, their chemotactic effect as extracellular proteins and the ability of anti-MT1/2 antibody to suppress leukocyte infiltration *in vitro* and *in vivo* indicates that MT1/2 might function as danger signals during colitis.

In **chapter 2**, we focused on the release of MT1/2 following apoptosis and necrosis since these are the two major forms of cell death implemented in the pathogenesis of human and murine intestinal inflammation. An increased rate of apoptosis was found in IBD patients and in DSS-induced colitis [5–7] and apoptotic cells become secondary necrotic cells when insufficiently phagocytised [8]. Epithelial necrosis has been described in experimental induced-colitis, in IBD patients and has even been demonstrated in crypts of patients without IBD [9–12]. Danger signals are per definition secreted by activated immune cells or passively released from dying cells following plasma membrane rupture. Plasma membrane rupture occurs during uncontrolled primary or secondary necrosis and in case of necroptosis and pyroptosis, two forms of programmed cell death. Necroptosis is a caspase-independent mode of cell death regulated by receptor-interacting protein kinases (RIPK)-1 and RIPK-3 and has recently been implemented in IBD pathogenesis [13]. Pyroptosis involves inflammasome and caspase-1 or capsase-11 activation and also results in cell swelling and plasma membrane rupture [14]. Inflammasomes are important mediators of intestinal inflammation and have genetically been linked to IBD [15]. We confirmed the presence of dying cells with plasma membrane damage in DSS and TNBS colitis and human ulcerative colitis by measurement of LDH activity in serum and H&E staining of colon sections (unpublished data, *Figure 8*). Of note, these techniques cannot distinguish between necrotic, necroptotic and pyroptotic cell death. Nevertheless, in our interest of DAMPs release, and thus potential MT release, they hold the same fate of plasma membrane rupture and cell lysis.

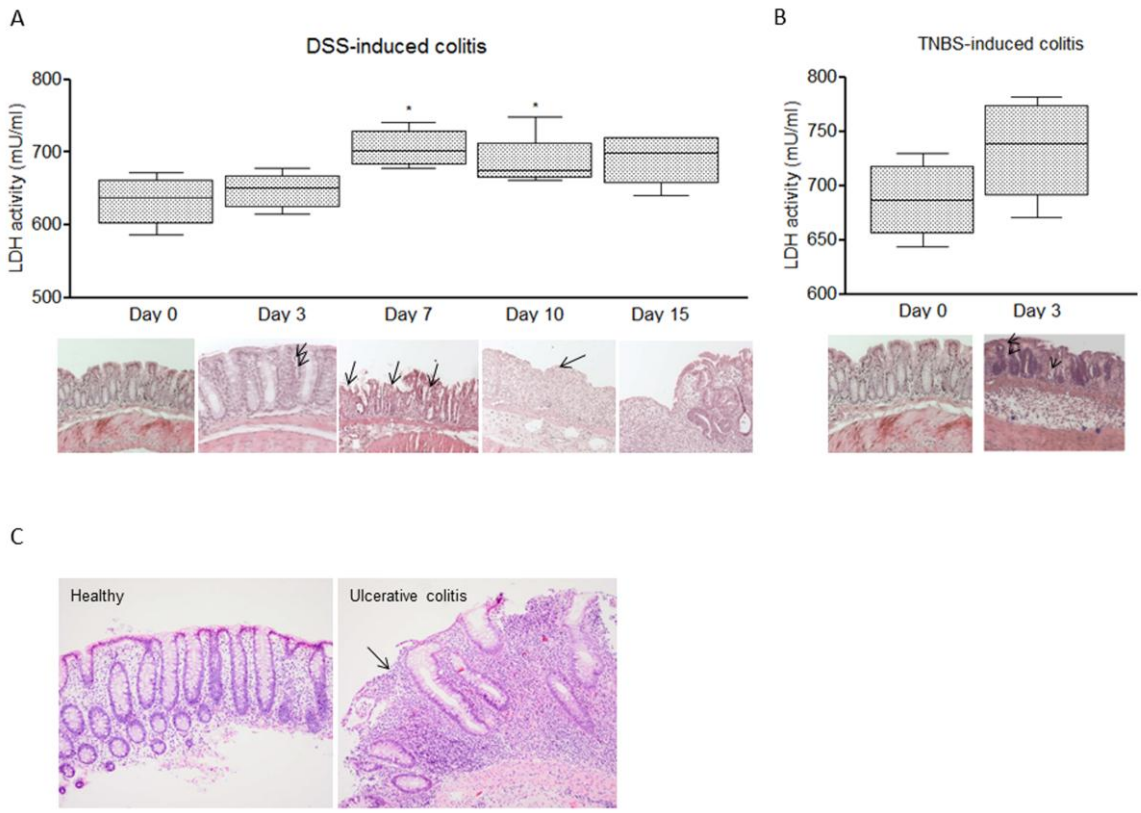


Figure 8: Colonic cell death is present during colitis. Lactate dehydrogenase activity (LDH) and representative images of H&E stained colon sections of mice during (A) DSS- and (B) TNBS-induced colitis. Data are represented as means ± SEM; n = 5 mice per time point; P < 0.05 compared to control (day 0). (C) Representative image of H&E stained colon section of a healthy control and ulcerative colitis patient. Arrays mark intact extracellular nuclei as residues of necrotic colonocytes. Original magnification x200.

The concept of targeting danger signals as treatment strategy in IBD has raised interest with the identification of HMGB1 as novel biomarker and potential target for IBD patients [16,17]. However, blocking one DAMP results in partial suppression of disease activity [8,16]. A more effective strategy could be the use of a multi-ligand receptor inhibitor. Potential therapeutic targets are RAGE and TREM-1, two specific DAMP receptors which signal via NF-κB activation upon ligand binding [18]. However, blocking RAGE or TREM induced signalisation also did not fully protect against experimental colitis [19–21]. Using multiple inhibitors of different ligands and receptors could be more effective but is challenged by finding a balance between the preservation of an essential immune response and the suppression of an excessive

inflammatory response. At the applied dose, anti-MT1/2 antibody therapy resulted in reduced leukocyte infiltration following DSS and TNBS treatment. If higher doses are able to enhance disease activity suppression while preserving effective immune responses will be tested in the future. Anti-MT1/2 antibody therapy might have some advantages compared to anti-TNF treatment, the most widely used antibody in the treatment of IBD. The biological function of MT1/2 is subtle (MT1/2 knockout mice have no adverse phenotype), whereas TNF is a central mediator of inflammation. Additionally, anti-MT1/2 antibodies might counteract the development of cancer since high MT1/2 levels have been identified as cancer risk factor in IBD patients [22]. However, anti-TNF used to be linked with increased risk of cancerogenesis but has recently been proposed as protective treatment in the development of colitis-associated cancer [23,24] and might in this aspect thus act synergistically with anti-MT1/2 antibodies. Notable, 10-30% of IBD patients does not respond to anti-TNF and this number increases to 40% because of loss of response over time. Thus, anti-MT1/2 antibodies might be an attractive strategy for end-stage IBD patients.

In **chapter 2** we showed that MT1/2 are absent in highly necrotic intestinal epithelium of active IBD patients. We speculate that (1) MT1/2 are released from the damaged intestinal epithelial cells and/or (2) the MT1/2 down-regulation is part of the protective HIF-mediated response (results of chapter 1). In order to strengthen the latter, double immunohistochemical staining for MT1/2 and HIF should be performed on human IBD samples. Notwithstanding this epithelial MT1/2 down-regulation, active IBD is associated with an infiltration of MT1/2 positive inflammatory cells which could also be a source of (massive) extracellular MT1/2 that can be targeted by anti-MT1/2 antibodies (see future perspectives). Antibodies act outside the cell and current results indicate that cell leakage is the only source of extracellular MT1/2. In this context, anti-MT1/2 antibody therapy might be most effective in active IBD patients, characterized by excessive cell death, in order to dampen on-going inflammation and in patients in remission to prevent the effect of released MT1/2 in a potential upcoming flare-up.

III. Metallothioneins and macrophage plasticity

Since the intestinal mucosa of IBD patients is infiltrated with MT1/2 positive macrophages and fibroblasts (unpublished data), we analysed the effect of MT1/2 on macrophage phenotype and polarisation in **chapter 3**. We report that MT1/2 deletion in murine bone-marrow derived macrophages (BMDM) is associated with reduced levels of M1 (TLR4/IRF5/IRF3) and increased levels of M2 (IL-4R/KLF4) signallers which resulted in a reduced pro-inflammatory response to LPS and an enhanced IL-4 response respectively.

The functional characterisation of macrophages is based on gene expression profiles and secretion of specific cytokines and chemokines. The classically M1 and alternatively M2 activated macrophages are involved in pro-inflammatory and immune suppressive mechanisms respectively [25]. In order to circumvent extravagant bystander damage, regulatory macrophages dampen immune activation and promote tissue restoration. However, M2 macrophages are also involved in wound healing and fibrosis, ultimately resulting in a deleterious outcome if uncontrolled. In optimal conditions , a subset of M2 macrophages seems to be involved in counteracting this process of fibrosis and promotes complete tissue recovery and homeostasis [26–29]. Colitis is associated with an M1/M2 imbalance and alternative M2 macrophages are associated with attenuated disease activity in experimental colitis [30,31]. Weisser et al. showed that M2 macrophages confer protection against DSS-induced colitis by using genetically modified mice with a pronounced M2 macrophage phenotype [30]. Moreover, transfer of *ex vivo* derived M2 macrophages was able to reduce DSS-susceptibility, demonstrated by two independent groups [30,31]. Thus, the reduced disease activity of MT-KO mice in DSS-induced colitis might additionally be mediated by reduced TLR4 and enhanced IL-4R levels in the macrophages of these mice. In order to strengthen these data, M1/M2 macrophage levels need to be determined in colonic tissue of healthy MT-KO and WT mice and in these mice during colitis.

Extrapolation of data derived from murine BMDM cannot simply be extrapolated to humans. Murine M1 and M2 macrophages can easily be distinguished based on gene expression profiles. Unfortunately , an unambiguous delineation for human macrophages is not present.

For example, the typical M2 polarization markers Arg-1, Chi3l3 (Ym-1) and Retnla (Fizz1) are not expressed by *in vitro* stimulated human macrophages. To circumvent this issue, we will further characterize the effect of MT1/2 on macrophage phenotype and function by using blood-isolated human monocytes. Previous research stated that there are two distinct macrophage populations in the gut, one derived from circulating mononuclear cells and the other comprised residing phagocytes [25,32]. This hypothesis has recently been refuted and Bain and Mowat claim that intestinal macrophages require constant replenishment from blood monocytes and differentiate in situ depending on the environmental stimulus [33]. In accordance with the latter paradigm, blood-isolated monocytes nicely represent the circulating pool of residing macrophages. The question remains however if these cells respond equally *ex vivo* as *in vivo* after entering the lamina propria.

In summary, colitis is characterized by necrotic cell death, which is associated with the release of MT1/2. Released MT1/2 attract leukocytes, a process that can be abrogated by anti-MT1/2 antibodies. Anti-MT1/2 antibody therapy partly suppresses experimental colitis and complete ablation of MT1/2 results in an even greater reduction of disease activity in MT1/2 knockout mice. These MT1/2 knockout mice harbour M2-skewed macrophages which is associated with an impaired pro-inflammatory and enhanced regulatory response following stimulation *ex vivo* and this could additionally have contributed to the reduced susceptibility to DSS-induced colitis in these mice.

Future perspectives

I. Metallothionein suppression in chronic IBD models

In **chapter 2** we showed that genetic deletion of *Mt1* and *Mt2* results in a significant reduced weight loss during DSS-colitis. Antibody-mediated MT1/2 inhibition reduces leukocyte infiltration with a marginal effect on weight preservation. The pronounced effect of genetic MT1/2 deletion compared to antibody treatment might result from the applied antibody dose and/or the additional effect of intracellular MT1/2 deletion on macrophage phenotype and function, described in **chapter 3**. To extrapolate anti-MT1/2 antibody therapy to clinical practice, additional experiments are necessary. Antibody dose effectiveness needs to be tested in acute and chronic colitis models which are more specific for the immune dysregulation in human IBD patients, such as the adoptive T cell transfer model. Secondly, safety and specificity of the anti-MT1/2 antibody should be determined. Systemically administered radioactively labelled anti-MT1/2 antibodies were detected at the inflamed colon on microSPECT/CT scans of mice at day 7 of DSS-induced colitis and colonic radioactivity was less pronounced in mice scanned during recovery. To be able to link SPECT/CT signals to colonic injury, every mouse was euthanized following scanning and the colon was removed for histological evaluation and auto-radiography. In order to evaluate anti-MT1/2 antibody accumulation and safety during colitis, mice should be injected at specific time points (extrapolated from pharmacokinetic studies), scanned through the course of colitis and euthanized when fully recovered. Additionally, colonic radio-activity following injection of radio-actively labelled anti-MT1/2 antibody during colitis should be compared to the activity observed after injection of radio-actively labelled IgG control antibodies. The observed enhanced colonic radioactivity following injection of radio-actively labelled anti-MT1/2 antibody could be specific and indicative for extracellular released MT1/2 in the colon but could also merely reflect increased intestinal vascularization during inflammation. Notably, irrespective of the latter outcome, the current results showed that the antibody at least reached the colon and could target MT1/2 during colitis.

Finally, our results implement the involvement of MT1/2 as pro-inflammatory mediators in the course of colitis. In order to comprise intestinal inflammation and IBD in general, the involvement of MT1/2 in ileitis needs to be determined. We already started a first pilot experiment by backcrossing Mt1/2-KO ($Mt^{-/-}$) mice to $TNF^{\Delta ARE/WT}$ mice (who have normal MT levels and are denoted as $Mt^{WT/WT}$,$TNF^{\Delta ARE/WT}$). The $TNF^{\Delta ARE/WT}$ mice are genetically modified mice which lack the RNA stability and transcriptionally-regulatory TNF AU-rich elements in one allele, resulting in an overproduction of TNF. These mice establish two specific phenotypes at the age of 6 to 8 weeks: chronic inflammatory ileitis and arthritis [34]. We followed up the first generation offspring of $Mt^{WT/WT}$,$TNF^{\Delta ARE/WT}$ x $Mt^{WT/WT}$,$TNF^{WT/WT}$ and $Mt^{WT/WT}$,$TNF^{\Delta ARE/WT}$ x $Mt^{-/-}$,$TNF^{WT/WT}$ mice and saw that $Mt^{WT/WT}$,$TNF^{\Delta ARE/WT}$ mice gained significantly less weight compared to their littermate controls $Mt^{WT/WT}$,$TNF^{WT/WT}$; while the $Mt^{WT/-}$,$TNF^{\Delta ARE/WT}$ mice showed the same weight evolution as their littermate controls $Mt^{WT/-}$,$TNF^{WT/WT}$ (*Figure 9A*). This effect disappeared after 16 weeks, which was, based on clinical evaluation of the mice and previously reports, presumable the result of MT1/2 deletion-associated obesity in the $Mt^{WT/-}$ mice on a C57BL/6 background [35]. Why $Mt^{WT/-}$,$TNF^{\Delta ARE/WT}$ do not develop this phenotype will be further investigated. Regardless of the disappearance in weight evolution, serum analyses revealed that $Mt^{WT/WT}$,$TNF^{\Delta ARE/WT}$ mice showed a higher increase in KC levels than $Mt^{WT/-}$,$TNF^{\Delta ARE/WT}$ compared to their respective littermate controls, whereas the opposite was observed for IL-4 levels (*Figure 9B*). These initial results are indicative for disease amelioration by partial MT1/2 deletion in $TNF^{\Delta ARE/WT}$ mice. To prove this hypothesis, weight evolution and additional parameters for ileitis will be compared between $Mt^{WT/WT}$,$TNF^{\Delta ARE/WT}$ and $Mt^{WT/-}$,$TNF^{\Delta ARE/WT}$ littermates. Breeding pairs delivering the appropriate littermates have been set up for future experiments ($Mt^{WT/-}$,$TNF^{WT/WT}$ x $Mt^{WT/-}$,$TNF^{\Delta ARE/WT}$). If results of MT1/2 deletion are favourable, anti-MT1/2 antibodies will additionally be tested in $TNF^{\Delta ARE/WT}$ mice.

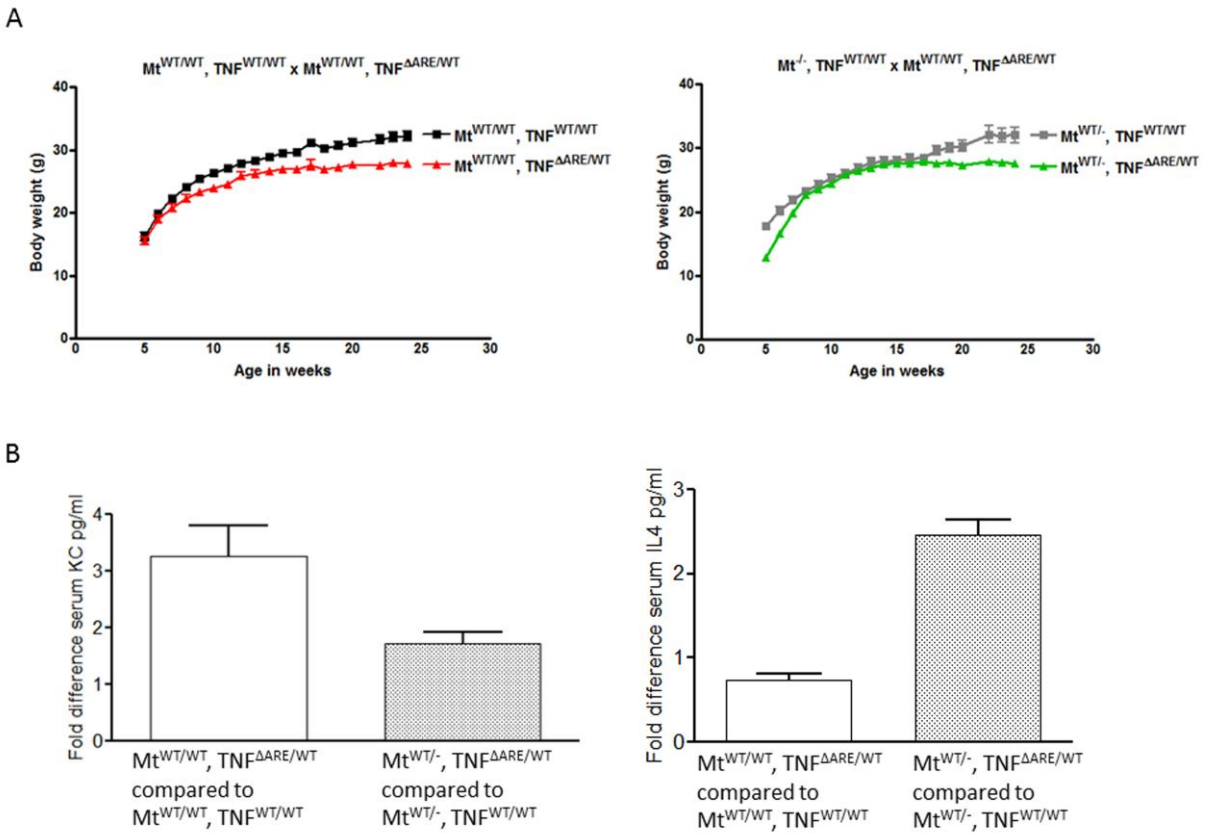


Figure 9: (A) Body weight evolution (n=5-12) and (B) fold difference of serum cytokine levels (n=3) of the first generation offspring of indicated breeding pairs. Cytokine concentrations were determined using luminex bead-based immunoassay.

II. Metallothionein suppression on macrophage function

1. Intracellular MT1/2 deletion

In **chapter 3**, we provide evidence for a differential expression in signal transducers and downstream effectors in WT and MT-KO macrophages. However, no conclusion can be drawn on the mechanism by which MT1/2 mediate this effect. Metallothioneins may directly regulate the transcription of TLR4/IL-4R and/or may exert its effect through downstream effectors that in turn control receptor levels.

Different transcription factors, such as NF-κB and KLF4, depend on intracellular zinc donation and MTs function as zinc chaperones. It has been shown that overexpression of MT1/2 results in reduced bio-available zinc levels whereas MT1/2 silencing increases free intracellular zinc concentrations in cancer cell lines [4]. While zinc signals are required for MyD88-dependent induction of pro-inflammatory cytokines [36], zinc negatively regulates TLR4 signalling through reduced IRF3 activation in macrophages [37]. Furthermore, zinc chelation/depletion elevated the oxidative burst but reduced the production of pro-inflammatory cytokines [38]. Apparently, low concentrations of zinc seem to stimulate TLR signalling while higher concentrations result in an anti-inflammatory effect of zinc on LPS-induced cytokine secretion [39]. Thus, zinc might be readily available in MT1/2 knockout macrophages and might as such participate in establishing the regulatory effect on macrophage phenotype. If zinc chelation in MT-KO macrophages results in an abrogation of the enhanced M2 phenotype/polarization, zinc can be addressed as a contributing causative factor. It is further to be investigated to what extent MT1/2 may function as selective zinc donator and thereby promote the M1 regulatory phenotype in macrophages.

The impact of MT1/2 deletion on macrophage phenotype and function may also participate in the reduced susceptibility of MT knockout mice to colitis. Bone-marrow derived macrophages from MT1/2 knockout mice display a regulatory phenotype and have a reduced LPS and an enhanced IL-4 response. TLR4 is essential for oral tolerance and bacterial defence [40,41]. However, excessive microbial infiltration due to intestinal barrier disruption might induce exaggerated TLR4 stimulation resulting in bystander damage during on-going inflammation. In addition, colonic epithelial proliferations and wound healing depends on Il-

4 and IL-13, cytokines associated with alternative macrophage activation [42]. In order to investigate if reduced TLR4 and enhanced IL-4 signalling might have attributed to the reduced signs of colitis in DSS-treated MT knockout mice, macrophage signatures need to be determined in colonic tissue of WT and MT-KO mice, at baseline and during colitis.

2. Extracellular MT1/2 suppression

In **chapter 2**, we showed that MT1/2 are released from necrotic IECs and attract leukocytes upon release. During intestinal inflammation, there is a high infiltration of MT positive inflammatory cells. Since danger signals leak passively from necrotic cells or are actively released from activated immune cells, we also investigated **if MT1/2 are released from macrophages**. We evaluated MT1/2 expression following LPS and IL-4 treatment and their subsequent release from M1 and M2 BMDM respectively. As previously described [43], we found an up-regulation of MT1/2 following LPS and not IL-4 (*Figure 10A*). However, MT1/2 could not be detected by western blotting in cell supernatant of BMDM following LPS nor IL-4 (*Figure 10B*). Similar data have been reported for DCs [44] and thus to date, no evidence exist for an active secretion of MT1/2 from immune cells. If in active IBD patients, highly MT1/2 positive macrophages undergo some form of cell death which results in cellular leakage of MT1/2 into the extracellular environment, they could serve as another source of extracellular MT1/2. Thus, other triggers which are known to release DAMPs from immune cells, such as inflammasome activation, should be tested for their ability to induce MT1/2 release from macrophages.

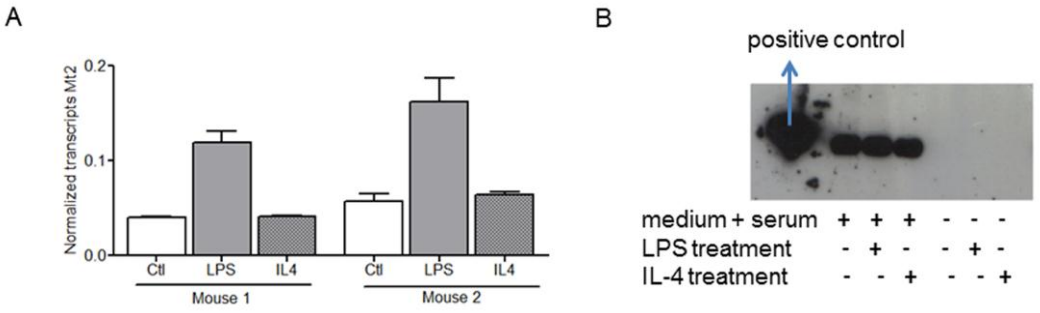


Figure 10: Bone-marrow derived macrophages were stimulated with 100 ng/ml LPS or 20 ng/ml IL-4 for 24 hours. The mRNA expression of Mt2 was analysed by qPCR; n=3 (A); supernatant was collected and analysed for MT by western blotting (B). Of note, MT secretion was tested using macrophage medium with and without foetal calf serum since this intrinsically contains high amounts of MT.

Extracellular MT1/2 aids in perpetuating the inflammatory response by attracting leukocytes [45,46]. The group of Lynes showed that MT binds the plasma membrane of macrophages and increases macrophage killing capacity upon phagocytosis [47]. We additionally investigated the effect of recombinant MT1/2 (Enzo Life Science, Antwerp, Belgium) on macrophage polarization. First results indicate that high doses of MT (10-30 µM) promote M1 signalization whereas low concentrations (0,1-1 µM) induce IL-4 secretion from wild type BMDM (*Figure 11A*). Anti-MT1/2 antibodies were able to block pro-inflammatory cytokine secretion following MT stimulation in BMDM (*Figure 11B*).

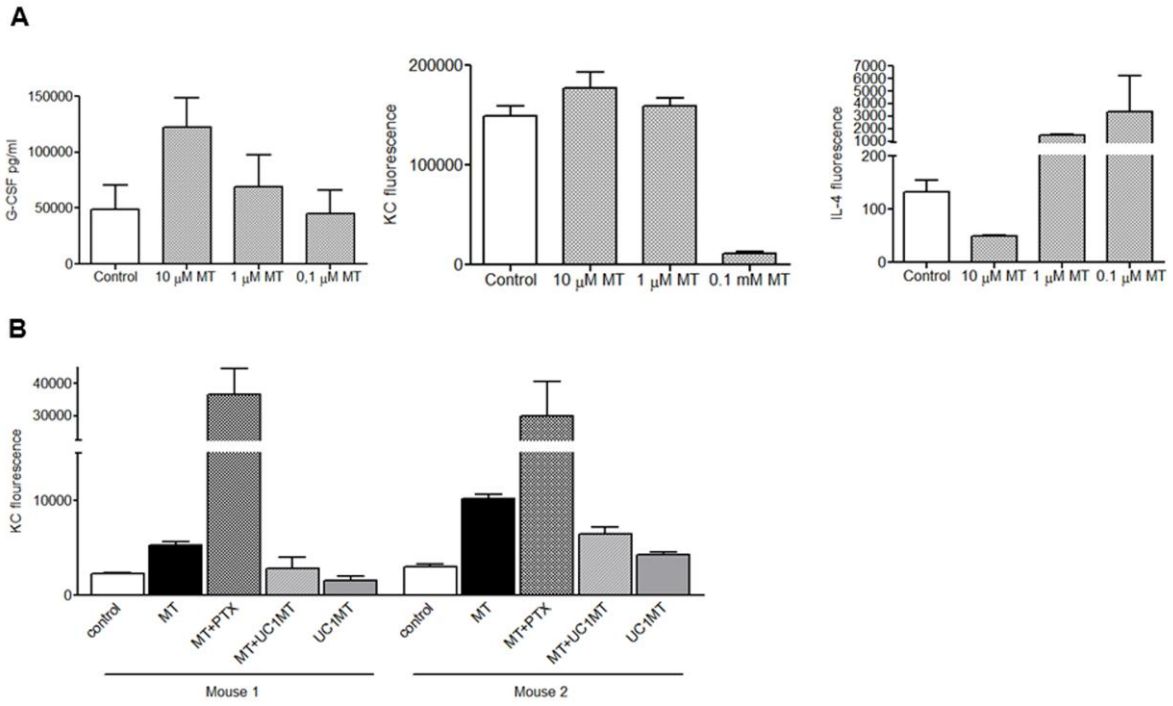


Figure 11: (A) Cytokine secretion of BMDM following different concentrations of recombinant MT1/2. (B) Supernatant KC cytokine secretion from BMDM following 30μM MT with and without 100μM pertussis toxin (PTX) or anti-MT1/2 antibody (130μg/ml UC1MT); n=2-3. Cytokine concentrations were determined using luminex bead-based immunoassay.

In future experiments, we will investigate how extracellular MT1/2 exert their action on macrophage polarization. A receptor for MTs has been identified on astrocytes using a fluoresceinated probe that binds all four MT isoforms [48]. In renal tubular cells, MT1 is believed to be internalized through megalin, a receptor for small protein ligands, whereas MT2 promotes neurite growth through the same receptor [49–51]. However, a specific MT1/2 receptor on macrophages has yet to be discovered. Leukocyte migration is mediated through G protein-coupled receptors (GPCRs) present on immune cells and can be blocked by pertussis toxin (PTX) [52,53]. The MT-mediated leukocyte migration could be inhibited by anti-MT1/2 antibodies and by PTX which may indicate that MT1/2 function through interaction with a GPCR [45]. On itself, PTX induces pro-inflammatory cytokine secretion in macrophages [54–57]. Additional treatment with PTX alone will have to reveal if the observed

marked increase in pro-inflammatory cytokine release following MT+PTX treatment results from an additive effect of MT1/2 and PTX on macrophage polarization (*Figure 11B*).

Additionally, TLRs are typical PRRs recognizing PAMPs but some of them also recognize endogenous danger-associated molecules. For example, HSPs are, like MT1/2, acute stress proteins denoted as danger signals and partly exert their pro-inflammatory action through TLR4 interaction [58]. If MTs also functions as TLR ligands and as such mediate M1 polarization is subject for further research. Future experiments employing specific inhibitors of M1/M2 signal transducers and transcription factors will reveal the mechanism by which MT1/2 drive M1 macrophage activation. The ability of high doses of recombinant MT1/2 to induce pro-inflammatory cytokine secretion further contributes to the denotation of MT1/2 as extracellular danger signals. Besides blocking chemo-attraction of inflammatory cells, anti-MT1/2 antibodies might be able to supress the pro-inflammatory effect of MT1/2 on macrophages and thereby limit the inflammatory response. To strengthen the hypothesis of MT1/2 as M1 stimulator and the potential to abrogate this by anti-MT1/2 antibodies, macrophage signatures need to be determined in intestinal tissue of anti-MT1/2 antibody and control treated mice during inflammation.

III. Metallothionein 3 as counterpart of MT1 and MT2?

Through this dissertation, we focussed on the role of MT1 and MT2 in experimental colitis and macrophage functional activation. Since MT3 is considered to be a brain specific isoform, its function has merely been investigated in neuronal disorders. However, we recently examined *Mt3* expression in IECs of mice during acute and chronic colitis and found that mRNA *Mt3* expression is, in opposite to *Mt1* and *Mt2*, decreased during acute DSS-induced colitis and is induced during chronic DSS-colitis (*Figure 12A*). This indicates that MT3 does not function as acute stress protein and presumably has a different, perhaps opposing, role in inflammation. In addition, MT3 expression was down-regulated following LPS stimulation whereas its expression was enhanced upon IL-4 treatment in murine BMDM (*Figure 12B*).

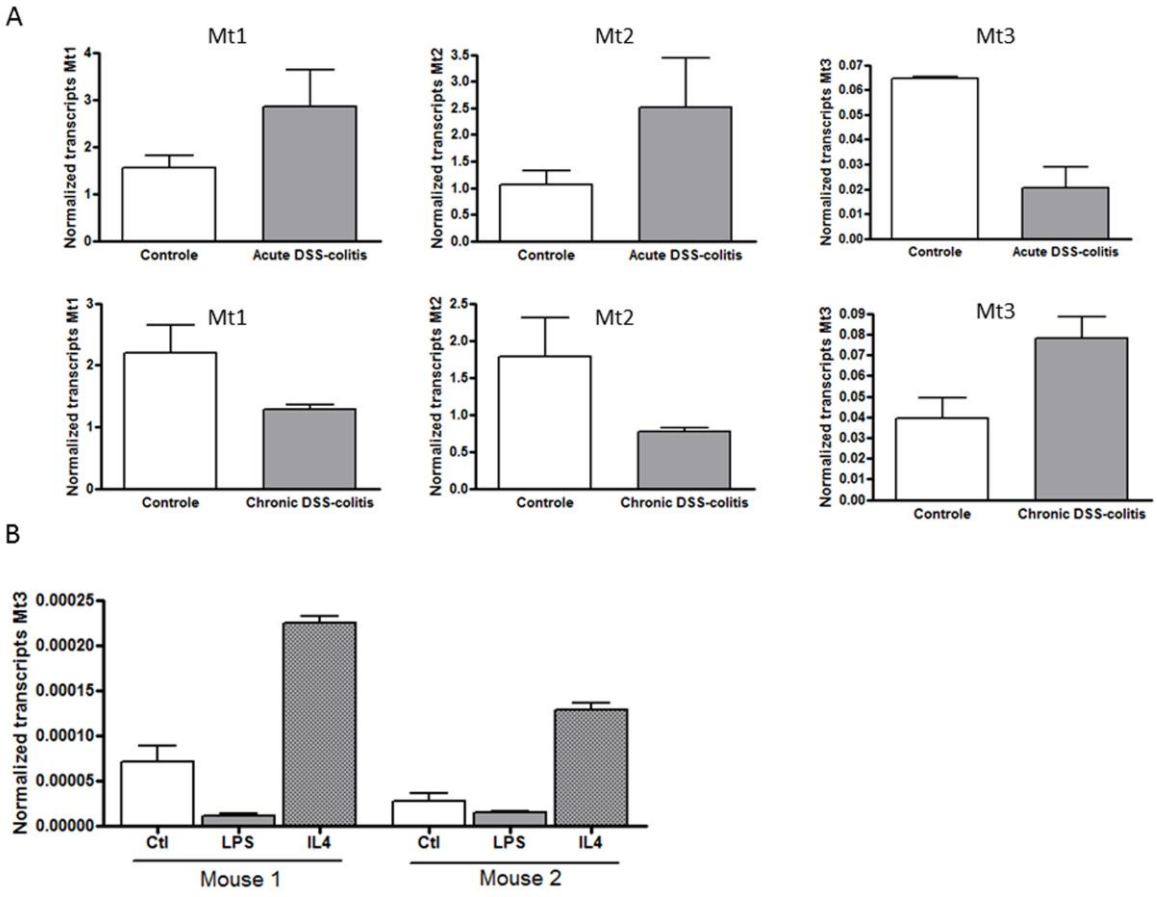


Figure 12: (A) Intestinal epithelial cells were isolated from wild type mice at day 7 of DSS-induced colitis and from mice following three cycles of DSS/drinking water. The mRNA expression of Mt1, 2 and 3 was analysed by qPCR; n=5. (B) Bone-marrow derived macrophages were stimulated with 100 ng/ml LPS or 20 ng/ml IL-4 for 24 hours, the mRNA expression of Mt3 was analysed by qPCR; n=3.

These results show that MT1/2 and MT3 are differentially expressed under inflammatory conditions and that MT3 associates with a regulatory macrophage phenotype.

Our current results show that MT1 and MT2 function as pro-inflammatory mediators and that targeting these two isoforms, genetically or by antibody therapy, reduces colonic inflammation. The preliminary opposing results for MT3 warrant for further investigation of the differential role of MT3 in the pathogenesis of intestinal inflammation and its potential therapeutic application in IBD.

Closing note

Inflammatory bowel diseases are characterized by a disruption of the intestinal epithelial barrier which is partly mediated by necrotic cell death. Necrosis results in the release of MT1/2 and other danger signals which attract and activate immune cells and further drive the inflammatory response (*Figure 13*). The use of anti-MT1/2 antibodies that specifically bind the inflamed colon represents an attractive strategy to suppress leukocyte infiltration and inflammation. Additionally, human colitis is characterized by an infiltration of MT1/2 positive macrophages and MT1/2 have been associated with a pro-inflammatory macrophage signature. It remains to be investigated if these MT1/2 positive cells are M1 macrophages and thus aid in perpetuating inflammation. Moreover, if extracellular MT1/2 is able to skew macrophages towards pro-inflammatory cells, the potential of anti-MT1/2 antibodies to counteract this polarization would strengthen their therapeutic use. Future research needs to confirm if anti-MT1/2 antibody therapy is able to reduce leukocyte infiltration and thus inflammation and additionally may aid in the process of mucosal healing and recovery by shifting the balance towards regulatory M2 macrophages in human IBD patients.

The results of this dissertation led to the filing of a patent application: 'Use of antagonists targeting metallothionein to treat intestinal inflammation' (TTRM PR2011/083 – P2010/099; national filing 17/01/2014). Future studies comparing anti-MT1/2 antibodies to conventional anti-TNF treatment in chronic IBD mouse models are in the pipeline to bridge fundamental, pre-clinical research towards the use of anti-MT1/2 antibodies in human clinical trails.

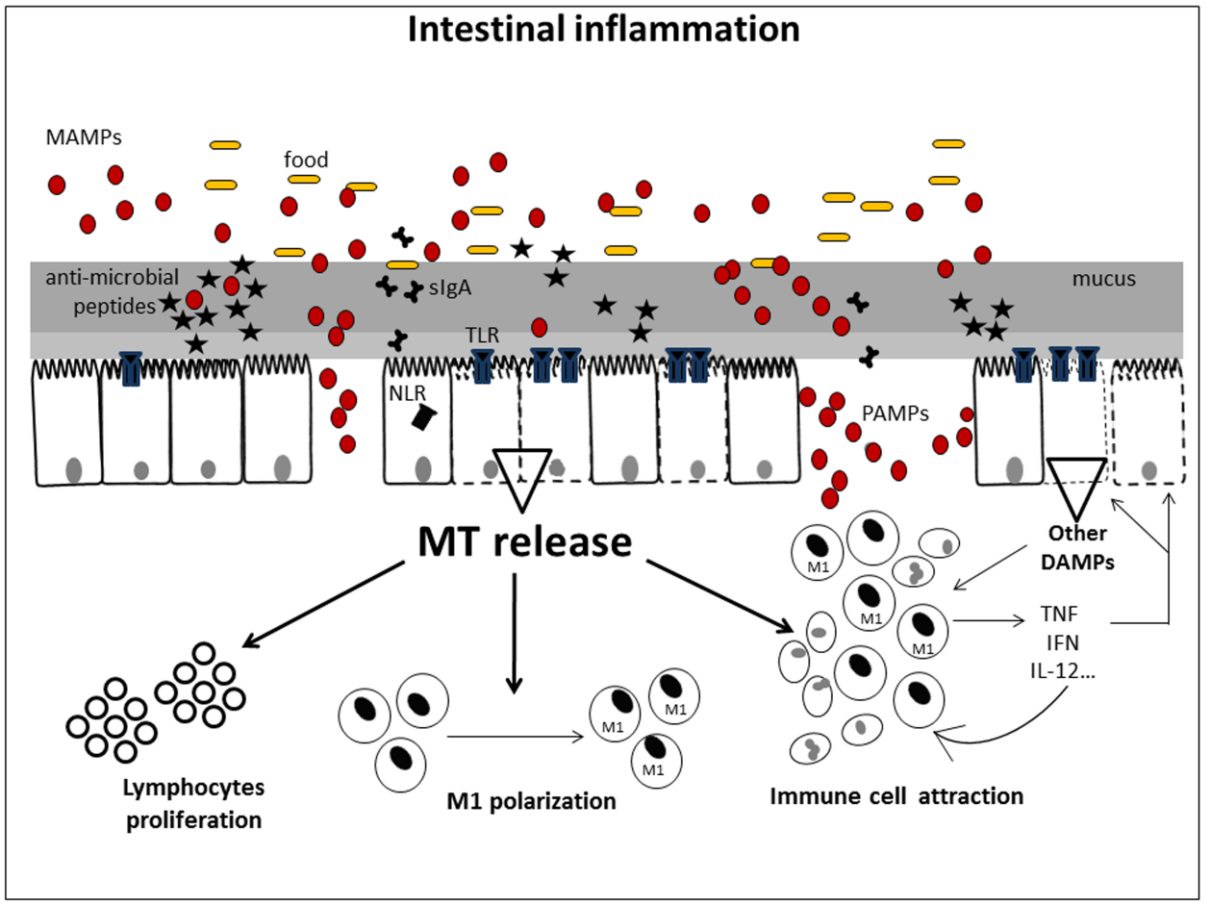


Figure 13: Overview of how metallothioneins (MTs) may drive intestinal inflammation. During intestinal inflammation, the epithelial barrier is compromised and pro-inflammatory cytokines and DAMPs, such as MT1/2, are released. Released MT1/2 attract leukocytes and polarize macrophages (M) towards a pro-inflammatory M1 phenotype. The latter contributes to more pro-inflammatory cytokine release and immune cell attraction which eventually results in bystander damage, further breakdown of the intestinal epithelium and maintenance of this viscous circle. IFN: interferon; IL: interleukin; MAMP: microbe-associated molecular pattern; NLR: NOD-like receptor; PAMP: pathogen-associated molecular pattern; TLR: Toll-like receptor; TNF: tumour necrosis factor. Adapted from Hindryckx and Laukens [59].

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Review activities

Reviewer for Clinical and Experimental Gastroenterology (2013)
Pharmacological Research (2012)

Patents

'Use of antagonists targeting metallothionein to treat intestinal inflammation' (TTRM PR2011/083 – P2010/099; national filing 17/01/2014)

Grands

2010-2014	PhD fellowship of the Scientific Research Foundations of UGent - Bijzonder Onderzoeksfonds, BOF
2011	Laureate of the yearly grant of the Flemish society for gastroenterology (Vlaamse vereniging voor gastro-enterologie, VVGE)

Student supervision

2013-2014	Lisa Crapé: Role of metallothioneines in experimental models for chronic intestinal inflamation, Master in Health Science and Medicine
2008	Rienske Mortier: Anaesthesia of the neonatal foal, Master in Veterinary Medicine

You cannot change the direction of the wind,

but you can adjust your sails to reach your destination.

(Adapted from Jimmy Dean.)

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The best and most beautiful things in the world cannot be seen or even touched - they must be felt with the heart.

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(Helen Keller)